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"A STUDY OF THE CHEMICAL COMPOSITION OF GRASS AND ITS CONSERVATION PRODUCTS"

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WILLIAM A. DEWAR A.H.-W.C., A.R.I.C.

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C O N T E N T S

PART I: DETERMINATION OF DRY MATTER AND VOLATILE COMPOUNDS IN SILAGE

C O N T E N T S (Contd.)

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PART I

DETERMINATION OF DRY MATTER AND VOLATILES IN SILAGE

INTRODUCTION

The conservation of fodder crops by ensiling is of increasing importance in this country. The method of ensilage involves the careful compaction of the crop and a rapid production of acidity brought about by the addition of acid solutions or preservatives or by the stimulation of lactic acid formation by bacteria present on the fresh material. The last-mentioned "natural" process is the commonest method in use in this country although a sugar additive in the form of molasses is frequently employed in order to present a readily available supply of energy material to the micro-organisms.

Although lactic acid production is the desired object of silage making by the natural process a number of other important biochemical and biological changes take place. Most of the soluble carbohydrate material, including glucose, fructose, sucrose and some oligosaccharides as well as fructosan, can be used as a readily available energy source by bacteria. Prior to bacterial fermentation, however, losses of sugars may occur due to the continued respiration of the plant cells with the production of carbon dioxide and water. This process depends largely on the existence of aerobic conditions and consequently on the degree of compaction during ensiling. Bacterial fermentation of soluble carbohydrates can produce a variety of organic acid by-products. Under good conditions lactic acid and acetic acid are the commonest of these acids but under certain other conditions large amounts of butyric acid may be produced along with

propionic acid and varying amounts of other fatty acids (Barnett 1954). Protein degradation also frequently occurs due to the action of both plant and bacterial enzymes (Macpherson 1952). In the early stages of breakdown amides and amino acids are produced while the prolonged action of bacterial enzymes under suitable conditions may result in the formation of ammonia and carbon dioxide. In addition to carbon dioxide and ammonia, hydrogen is sometimes produced during ensilage (Mabbit 1952). The formation of these gases represents an important loss of dry matter. Apart from this gaseous loss valuable nutrients flow from the silo in the effluent, the extent of the loss being dependent on many factors but chiefly on the original moisture content of the ensiled crop.

The dry matter losses occurring as a result of the above changes can vary considerably and losses of up to 23 per cent have been reported by Watson and Nash (1960). The dry matter loss is of extreme importance in assessing the value of any ensilage technique and it follows that the dry matter determination is one of the most important determinations carried out in a silage balance experiment. Experiments of this type have been performed by a number of workers and have been reviewed by Watson and Nash (1960). More recently McDonald and Attwood (1958) have described silo equipment which allows up to a ton of grass to be weighed to the nearest 100g, corresponding to a sensitivity of 1 in 25,000. There is therefore a need for a method of dry matter ~~of~~ determination of a high degree of accuracy for use in such experiments.

Methods of Determining Dry Matter

a) Oven drying

The most common method of determining dry matter is by heating a weighed sample of the material under test in an oven until it reaches constant weight. The loss of weight is considered to be moisture and the residue dry matter. While the method is considered to be reasonably accurate for most feeding stuffs including grass it is subject to error when used for silage which contains a number of volatile materials not present in the original grass. During oven drying these substances escape along with water giving rise to a falsely low dry matter value. In the past this error has frequently been neglected in silage studies and in the opinion of Perkins (1943) many of the reported dry matter losses occurring during ensilage have been overestimated because of failure to correct this error.

Apart from volatile loss there are a number of other factors which affect the accuracy of the method. For example there has been much discussion on which is the most suitable temperature for drying plant material. Nelson and Hulett (1920) found that the amount of water lost from an organic substance is dependent upon the drying temperature used. A sample which has reached constant weight at one temperature may lose more weight at a higher temperature. This further loss of weight is often attributed to the loss of gaseous or volatile products of the decomposition of the dry matter, including water which has previously formed an integral part of an

organic compound, but it may be partly due to the release of what has been called "colloid water". This is the water associated with various materials such as proteins, polysaccharides and lipids which are present in the plant in the colloidal form. Common (1951) has pointed out that because of the complexity of biological material there is likely to be a considerable variation in the intensity with which water is held by colloids; such intermolecular forces as hydrogen bonding are involved. It seems unlikely therefore that all the "colloid water" will be lost at the same temperature when plant material is heated. For this reason it is difficult to make a clear distinction between "colloid bound" and other forms of water occurring in the plant.

A temperature of 100°C is commonly used for drying. It is sometimes suggested that this temperature is too high for drying plant material and that decomposition of organic matter is likely to occur. Parish and Robinson (1951), however, have stated that enzyme activity is not suppressed in herbage until a temperature of 80°C has been reached. It is therefore desirable that this temperature should be quickly exceeded in order that dry matter losses due to enzymolysis may be minimized. Raymond (1951) has found that a forced draught drying oven at 100°C is suitable for this purpose. Similar ovens at lower temperatures take too long to raise the temperature of the sample during the initial stages of drying.

Willets (1951) has mentioned that the physical structure of the

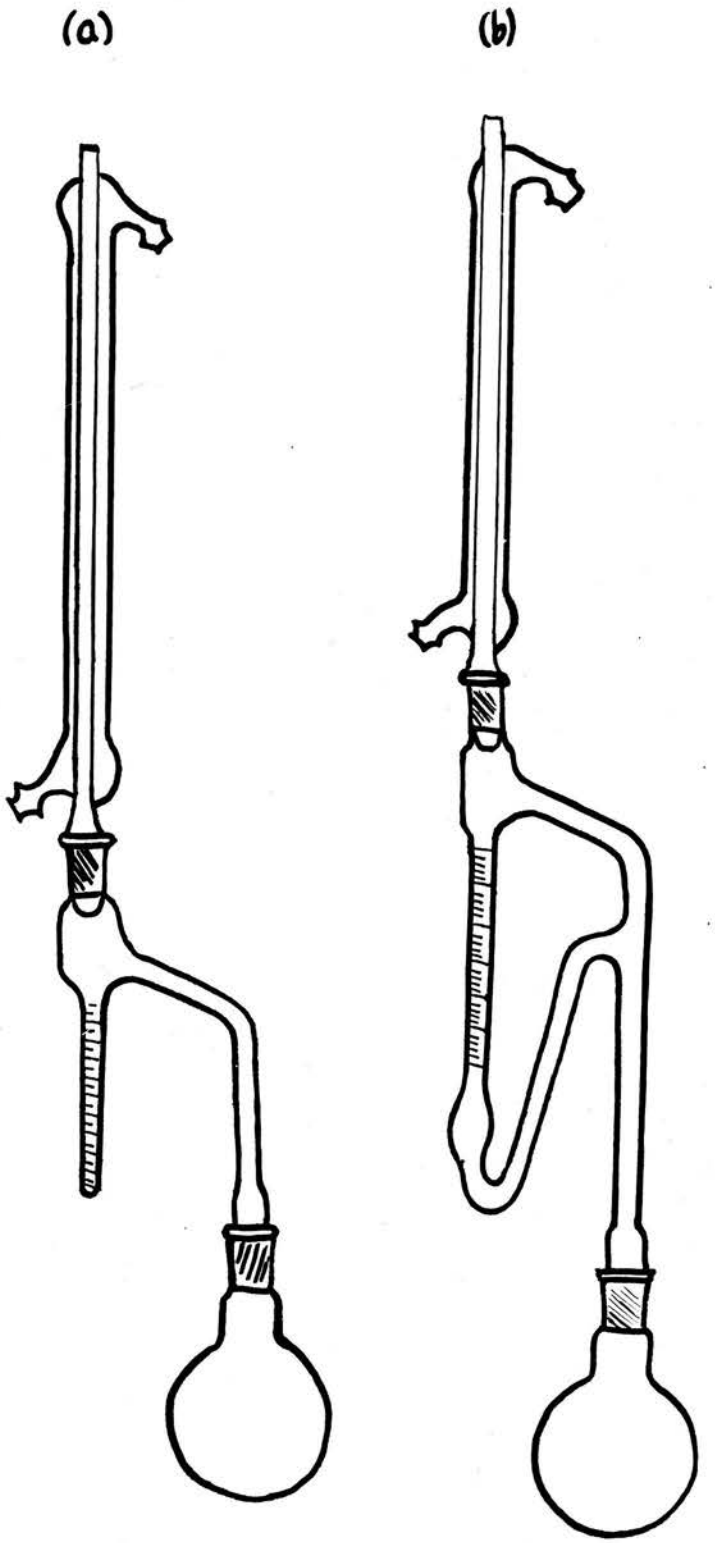

Fig. 1. Apparatus for determination of water by distillation with an immiscible liquid.

plant tissue may also be a contributory factor to errors in drying. During the drying process, as water is lost, the cell fluid becomes more concentrated and there is a lowering of the vapour pressure of the residual water; in addition heating often makes the cell wall less permeable to water. The result is that deep seated moisture may be difficult to remove in the later stages of drying. In order to avoid this error it is advisable to have the sample in as fine a state of division as possible.

b) Distillation with a liquid immiscible with water

In this method the sample under test is heated in a distilling flask with an excess of a liquid which has a boiling point near to that of water and is immiscible with it. The water and immiscible liquid are distilled over and after condensing are collected in a graduated receiver or trap which is so designed that the excess immiscible liquid flows back into the boiling flask. The distillation is continued until all the water has been driven off from the sample and the volume of the water in the trap is then measured. Two types of apparatus are shown in Fig. 1. The trap in Fig. 1a was designed by Bidwell and Sterling (1925) for use with liquids lighter than water while the trap in Fig. 1b is suitable for use with liquids heavier than water and is based on the original design of Langeland and Pratt (1938). Methods using the latter type of trap suffer from the disadvantage that the heavy liquid has to pass through the water layer before returning to the flask thus increasing the chance of emulsion formation. Further considerations will therefore be

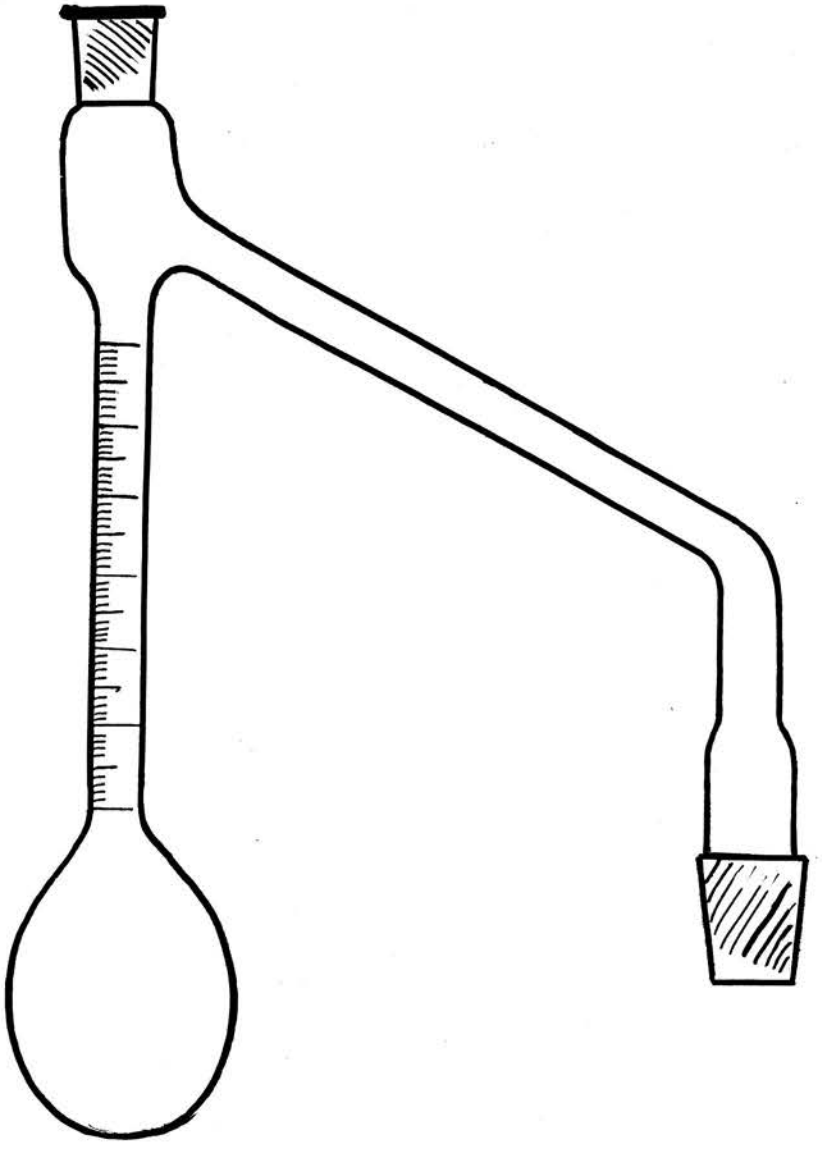

Fig. 2. Evans and Fetzer trap for use with a distilling liquid lighter than water.

confined to the method using liquids lighter than water.

The accuracy of the method is largely governed by the accuracy with which the volume of the water may be measured in relation to the sample size. As a certain minimum bore of trap is required for a good separation of water to take place a restriction is necessarily imposed on the accuracy with which the volume scale may be calibrated. On a trap of minimum bore, having a capacity of about 8 ml. the smallest division on the volume scale is usually 0.1 ml. but it is possible to read to the nearest 0.05 ml. This size of trap is suitable for fresh forage samples of about 10g. Assuming that such a sample contains 2g. of dry matter the error in calculating the dry matter percentage would be $\frac{0.05}{2} \times 100$ or 2.5% of the dry matter. Traps of about 80 ml. capacity, suitable for use with 100g samples, are of a correspondingly wider bore and may be read to the nearest 0.5 ml. The error involved is therefore similar to that in the smaller trap.

The accuracy of the method was improved by Evans and Fetzer (1941) who increased the capacity of the smaller size of trap by attaching an ovoid shaped bulb to its lower end (Fig.2). In this way it became possible to use a larger sample while maintaining the accuracy of volume measurement previously available for the smallest size of sample. The ovoid shape of the bulb was chosen to prevent occlusion at the junction of the bulb and graduated stem.

The distilling liquid most commonly used for plant materials is toluene (b.p. 110°C). When applied to cereals and fodder crops

with a low volatile content the toluene distillation procedure has been found to give dry matter values close to those obtained by oven drying (Sair and Fetzer 1942, Perkins 1943).

The distillation method possesses an advantage not shared by oven drying in that any volatile compounds lost during the distillation and passing in to the aqueous distillate may be determined and allowance made for the volume occupied by them.

c) Other methods

One of the most widely used methods of determining moisture is that devised by Karl Fischer (1935) in which the water is extracted from the sample by means of a solvent such as anhydrous methanol and then determined by titration with Karl Fischer Reagent (a mixture of iodine, sulphur dioxide, pyridine and methanol). The method has been used with some success for determining the moisture content of cereals and cereal products (Fosnot and Haman 1945). Unfortunately it is not likely to be suitable for determining the moisture in silage because of the possible presence of interfering compounds such as amines, carbonyl compounds and ascorbic acid.

Moisture meters, which depend on the measurement of electrical conductivity or dielectric constant, work best in materials which are uniform in particle size and composition (Grover 1946) and cannot therefore be expected to give good results with silage because of its heterogeneous nature.

TABLE 1

The volatile fatty acids present in field silage

Average pH	pH range	Total volatile fatty acids as percentage of dry matter	Individual volatile fatty acids as percentage of total acids							
			C_1	C_2	C_3	n-C_4	n-C_5	n-C_6	n-C_7	n-C_8
3.77	3.60 - 3.80	2.67	1.1	77.6	11.8	9.3	0.3	-	-	-
3.90	3.81 - 4.00	3.23	1.5	77.8	9.0	11.8	0.2	-	-	-
4.17	4.01 - 4.20	2.01	-	66.1	15.1	18.7	-	-	-	-
4.29	4.21 - 4.40	1.89	1.2	70.2	14.3	11.7	2.7	-	-	-
4.51	4.41 - 4.60	1.21	6.7	79.0	5.4	8.9	-	-	-	-
4.70	4.61 - 4.80	4.47	3.3	50.0	8.7	27.9	2.2	7.8	-	—
4.90	4.81 - 5.00	4.11	-	49.4	5.5	13.0	2.0	18.7	2.6	2.7
5.12	5.01 - 5.20	7.18	1.2	51.4	8.8	30.1	5.0	3.8	-	-
6.38	5.21 - 6.40	0.90	-	36.7	11.9	34.9	12.7	3.9	-	-
6.98	6.41 - 7.20	0.64	-	89.9	4.7	5.4	-	-	-	-

The Occurrence and Origin of Volatile Materials in Silage

a) Volatile fatty acids

The volatile fatty acids are the lower numbers of the fatty acid series beginning with formic acid and ending arbitrarily with capric acid. Although all of the acids have boiling points in excess of 100°C they are volatile in steam and must therefore be considered as potentially volatile during the drying of a watery material such as silage. Indeed their steam volatility has been shown by Duclaux (1874) to increase with increasing molecular weight.

Volatile fatty acids were first identified in silage by Russell (1908) when he analyzed the steam distillate from 10 kg. of silage. He found that acetic and butyric acids were present in the greatest quantities and that small amounts of formic, valeric, hexoic, caproic and isopropylacetic acids were also present. The separation of the volatile fatty acids was until recently a matter of some difficulty. The distillation methods of Wiegner (1926), Dyer (1917), and Lepper (1937) permitted the amounts of acetic and butyric acid to be calculated by the use of formulae but made no allowance for the presence of other acids. Since the advent of chromatography, however, a number of reliable techniques have been introduced for the separation of these acids with the result that information is now available about their distribution and occurrence in silage. Table 1 (Barnett 1954) shows the average figures, derived from about 50 samples, for the individual volatile fatty acids present in field silage. These figures are expressed as percentages of the total

volatile fatty acids present and are grouped according to the pH of the silages.

Acetic acid occurs in all types of silage and is produced by many micro-organisms including some lactic acid bacteria (e.g. certain strains of lactobacilli, streptococci and pediococci) and also by some coliform bacteria. Butyric acid, on the other hand, is a product of the bacterial breakdown of lactic acid by members of the group Clostridia (van Beynum and Pette 1936). There is also some evidence that it may be derived directly from disaccharides by saccharolytic bacteria (Barnett 1954). The acid is frequently found in silages with high pH values. Little is known about the conditions responsible for the formation of propionic acid in silage although van Beynum (1937) has suggested that it is formed in large quantity when the pH falls below 4.2. The higher volatile fatty acids are usually found only in poorly preserved silages and there only in small amounts. Barnett (1954) has proposed that these higher volatile acids are products of the deamination of amino acids by bacteria.

b) Lactic acid

Lactic acid is the chief acid found in well preserved silage and its production by lactic acid bacteria, chief of which are the lactobacilli, is responsible for the low pH value of good silage. Lactic acid is frequently considered to be non-volatile but its partial volatility in steam was demonstrated by Woodman (1925) when he evaporated an aqueous solution of the acid in a steam oven. An interesting feature of this experiment was the fact that part of the

residual lactic acid had undergone chemical transformation during heating and was present in the form of lactic anhydride which could be reconverted to the original acid by hydrolysis with sodium hydroxide. The steam volatility of lactic acid has since been confirmed by Smith (1938) who found that about 5% of the acid was volatile during steam distillation. When it is considered that lactic acid often constitutes up to 10% of the dry matter of silage even a slight volatility may be significant in affecting the dry matter value obtained by the oven drying method.

c) Volatile bases

The volatile bases in silage are present as a result of protein degradation. The proteins are first broken down to amino acids which under suitable conditions may be deaminated by bacteria with the formation of volatile bases. The nature of the volatile nitrogenous compounds present in silage has been examined by Macpherson (personal communication) who concluded that ammonia was the main constituent. Watson and Ferguson (1937) found that the volatile bases in a number of silages they examined were present in largest quantities in silages with high pH values. Even at pH values above 5 there are usually sufficient quantities of organic acids in the silage to ensure that the volatile bases will be present in the combined state.

d) Alcohol

The presence of alcohol in silage has been reported by a number

of workers including Hart and Williman (1912), Peterson et al (1925) and Mabbit (1952). It is not clear whether alcohol is produced during the early stages of ensilage due to respiration or whether it is present as a by-product of microbial action. The average alcohol content is quoted by Watson and Nash (1960) as being 0.3% of the fresh silage.

e) Esters

When alcohol is present in silage the possibility exists that it will react with one or more of the organic acids present to form an ester or esters which may be volatile. The quantity of ester formed will depend upon the conditions existing in the silage but because esterification is reversed by the presence of water it is unlikely that the reaction will go to completion. Consequently the presence of an ester in silage is always likely to be accompanied by the presence of alcohol.

Methods for Correcting Silage Dry Matter for Volatile Loss

The difficulty in obtaining a true figure for silage dry matter, because of the loss of volatile materials during drying, has long been recognized and several attempts have been made to assess the magnitude of the error involved. The methods used fall into two categories.

a) By analysis of the fresh and dried silage

In this method the volatile materials are determined in the fresh

silage and again in the silage after drying, the difference being regarded as volatile loss. Only the loss of volatile acids and bases has been investigated.

Using this method Crasseman (1924) reported an average loss of volatile acids amounting to 1% of the fresh silage on drying in a vacuum oven at 60°C. Woodman (1925) encountered a loss of volatile acid (expressed as acetic acid) of 1.25% and an ammonia loss of 0.17% of the fresh silage using a steam oven

Watson and Ferguson (1937) examined 67 samples of silage before and after drying at 98°C for total volatile acid content which was expressed as acetic acid. The loss of volatile acid ranged from 50.1 - 90.2% with a mean value of about 77%. The loss of volatile bases was also determined. Correction of the dry matter figures was carried out in each case by adding on the total loss per 100g. of fresh silage to the percentage apparent dry matter. This method of correction has been used in studies by Schoch (1949) who used a drying temperature of 60 - 65°C for 24 hours followed by 3 hours at 105°C and by McDonald and Purves (1956) at 100°C.

More recently Colovos, Keener and Davis (1957) have calculated the total volatile loss in terms of energy by carrying out energy determinations on silage before and after drying.

b) By using the toluene distillation method

Perkins (1943) determined the dry matter values of a number of silages by the toluene distillation method and found that in every case they were higher than the corresponding figures obtained by

oven drying at 100°C. Having established that the two methods gave almost identical results when used for crops containing no volatiles, Perkins suggested that by simultaneous application of the two methods to silage a measure of the amount of volatile compounds present in the silage could be obtained. This would certainly be valid if the volume of water in the toluene method contained no volatiles but quantities of acid up to 0.07g. in weight were found in the distillates from 10g. of silage. The effect of this acid on the volume of the water was ignored by Perkins because its volume was smaller than the smallest division on the volume scale. Even if the volume of the acid was as little as 0.05 ml. the resulting error would have been 0.5% of the fresh silage or about 2.5% of the dry matter.

In spite of this obvious error the toluene method has been used without correction in silage studies by Archibald (1946), Wittwer et al (1958) and Langston et al (1958).

Object of Present Investigation

The purpose of the present investigation was firstly to determine the nature and quantities of the volatile compounds lost on drying silage and secondly to develop a more accurate method than at present exists for the determination of silage dry matter.

The existing method of assessing the loss of volatile constituents depends on the analysis of the fresh and dried silage. Only volatiles which are not involved in any chemical reaction

during the drying process may be examined in this way. The discovery of Woodman (1925) that lactic acid is partially changed to lactic anhydride at 100°C makes it clear, therefore, that the method is unsuitable for determining the volatile loss of lactic acid. Similarly, the volatile loss of alcohol cannot be determined by this method as alcohol may take part in esterification with organic acids during drying. Provided that the esters produced are volatile no great error should occur. But formation of a non-volatile ester would lead to an overestimation of the amount of the alcohol lost.

It was therefore decided to adopt a new approach to the problem by attempting to collect and analyse the water and volatiles lost on drying silage at 100°C. In this way it was hoped to obtain information on the volatile losses of lactic acid and alcohol, which have not previously been measured, as well as on the losses of the volatile fatty acids and volatile bases.

EXPERIMENTAL METHODS

Construction of Oven Distillation Apparatus for the Determination of Dry Matter

The first object of the experimental work was to construct an apparatus in which the conditions in a forced draught drying oven at 100°C could be simulated and in which the water and volatile compounds lost on drying silage could be recovered quantitatively for analysis.

Preliminary experiments on a conventional glass distillation apparatus, capable of holding 100g. of silage, suggested that this might be suitable for the purpose provided that the following requirements could be fulfilled:-

1) the distillation flask could be maintained at a steady temperature of 100°C for about 18 hours;

2) a current of carbon dioxide and water free air could flow through the distillation flask at a similar rate to that used in a forced draught drying oven;

3) the water and volatiles lost from the silage could be efficiently condensed from the air stream and collected in such a way that they would be available for analysis.

In a large forced draught drying oven the temperature of the air is thermostatically controlled and remains steady at 100°C for long periods of time. In order to produce similar conditions in the apparatus it was essential that the distillation flask should be externally heated to 100°C so that the incoming air at 100°C would not

be subjected to any heat loss. This was achieved by enclosing the distillation flask in a small thermostatically controlled electric oven set at 100°C.

The preheating of the air stream was effected in the same oven, in which, because of the situation of the heating elements, the temperature of the oven floor was 135°C. By passing the incoming air through a coil of copper tubing resting on the oven floor it was possible to raise the temperature of the air stream to 100°C. It was found that a rate of air flow of 14 litres per hour at 100°C was required to dry 100g. of silage to constant weight in a similar time (18 hours) to that taken by a forced draught oven at the same temperature.

A small electric air pump of the vibrating diaphragm type was used as a source of air supply. Before entering the oven the air was freed from atmospheric moisture by passing it through a calcium chloride tower and then through concentrated sulphuric acid which also served to remove any ammonia present. Removal of carbon dioxide was then carried out by means of soda lime.

The complete condensation of water and volatiles from the air stream presented some difficulty in experiments in which solutions of volatile compounds were evaporated in the distillation flask. A water condenser alone was not satisfactory. While cooling the receiving flask to temperatures of about -20°C improved the efficiency of condensation, the flow of air through the apparatus was stopped after several hours due to the system being blocked by the formation

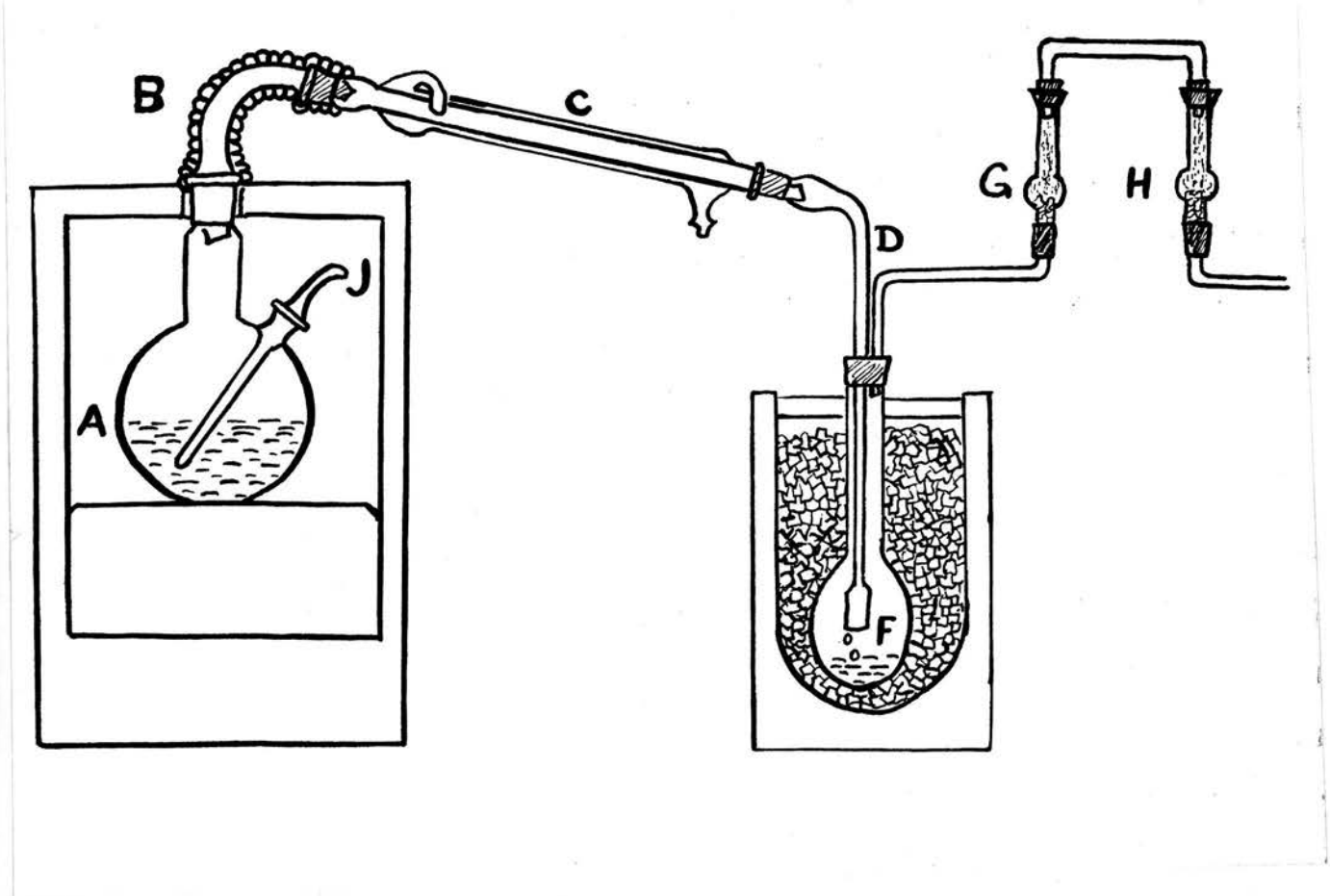

Fig. 3. Oven distillation apparatus for the determination of dry matter and volatiles in silage.

of ice in the delivery tube leading to the receiving flask. A slightly higher cooling temperature (-10°C) was therefore used and the remaining traces of water were removed from the air stream by a weighed absorption tube containing silica gel which was chosen in preference to other desiccants because of its inert chemical nature. Volatile compounds trapped along with water in the absorption tube could therefore be eluted for determination.

Also included in the system were a weighed soda lime absorption tube to detect the presence of any carbon dioxide resulting from the decomposition of organic matter and a gas wash bottle containing standard sulphuric acid solution to trap any remaining ammonia from the air stream.

The essential features of the apparatus in its final form are shown in Fig. 3. The dry, carbon dioxide free air preheated to 100°C enters the round-bottomed, two-necked 1000 ml. flask (A) through the side-neck (J). The main outlet of the flask is connected through an insulated stillhead (B) passing through an opening in the oven roof to a Liebig condenser (C) and thence through a long delivery tube (D) into a 300 ml. Kjeldahl flask (F) which acts as a receiver. During a determination the flask is enclosed in a wide-mouthed vacuum flask containing a mixture of crushed ice and salt at -10°C. The air leaves the apparatus through preweighed absorption tubes containing silica gel (G) and soda lime (H). Finally the air passes through a gas wash bottle (not shown in the diagram) containing standard sulphuric acid solution.

Notes on the apparatus

1. Ordinary rubber tubing was found to be unsatisfactory for use inside the oven at 100°C as it soon deteriorated and allowed leakage of air to take place. The tubing leading into the copper coil and connecting it to the apparatus was therefore made of heat resistant silicone rubber.

2. Condensation of water in the vertical part of the stillhead, which slowed down the removal of water from the distillation flask, was minimized by lagging the stillhead with asbestos string.

3. The inner surfaces of the Liebig condenser and delivery tube were made water-repellent by treating them with a silicone compound (Silicone Fluid MS 1107 manufactured by Midland Silicones Ltd.). Water thus ran quickly down the surfaces in the form of discrete drops and adhesion of water to the glass was reduced to a minimum.

4. The lower two inches of the delivery tube leading into the receiving flask were of a wider bore than the rest of the tube to reduce the risk of the tube being blocked by frozen condensate.

5. The rate of air flow was measured by means of a simple flow meter of the type described by James and Martin (1952) in which the passage of a bubble of sodium stearate up a tube of known volume is timed. By measuring the rate of air flow before and after it had passed through the oven distillation apparatus it was possible to discover any leakage of air.

Methods of Chemical Analysis

Volatile fatty acids

The column chromatographic procedure of Wiseman and Irvin (1957) was adopted for the separation and determination of the volatile fatty acids. This method, which was developed specially for silage studies, has the virtue of requiring very little sample preparation; in addition, as it employs an internal indicator, there is no need for mechanical fraction collecting equipment. Celite, stabilized with sugar solution, is used as adsorbent and mixtures of acetone and petroleum ether (b.p. 60° - 80°C) as eluents. Formic, acetic, propionic and butyric acids may be determined after separation on the columns, while higher acids may also be separated when present at a favourable level. The acids are added to the column in a dilute sulphuric acid solution and after separation are titrated with standard alkali solution.

Wiseman and Irvin succeeded in obtaining a separation of lactic and succinic acids on the column but the author experienced difficulty in obtaining a quantitative elution of these acids in spite of the use of Celite which had been purified by washing with hydrochloric acid. The method was therefore used only for the determination of the volatile fatty acids and lactic acid was determined by another method.

Lactic acid

Lactic acid was determined by the method of Elsden and Gibson

(1954). The lactic acid in N sulphuric acid solution is oxidised by means of ceric sulphate solution to acetaldehyde in a specially designed steam distillation apparatus. The acetaldehyde is removed by steam before further oxidation to acetic acid can take place, and is absorbed in excess sodium bisulphite solution and then determined in the usual way. Proteins and sugars interfere with the reaction and are removed at the beginning by precipitation with tungstic acid and cupric hydroxide respectively.

Blank determinations, carried out without the addition of ceric sulphate, serve as an indication of the amounts of aldehydes and ketones which may be present in the test solution.

Alcohol

Alcohol was determined by the microdiffusion technique of Winnick (1942) in which the alcohol diffuses from the outer compartment of a Conway dish to be absorbed and oxidised by standard potassium dichromate in sulphuric acid solution. The amount of potassium dichromate required is calculated after a back titration and hence the amount of alcohol in the sample. The method is not specific for ethyl alcohol, and other alcohols interfere as do aldohydes and ketones. Some measure of the latter substances may be obtained from the blank determinations in the lactic acid method and the necessary corrections may therefore be made.

Nitrogen and Ammonia

Nitrogen was determined by the Kjeldahl method, as modified by

Ashton (1936), using selenium as catalyst. Ammonia was determined in Markham's micro-Kjeldahl steam distillation apparatus.

pH Value

The pH value of the silage was determined in an aqueous extract of the fresh silage by means of a glass electrode pH meter.

Experimental details of the above methods together with the results of recovery tests carried out on pure solutions and silage extracts are given in Appendix II.

Preliminary Tests on Oven Distillation Apparatus

Pretreatment of air supply

The efficiency of the pretreatment of the air supply in removing atmospheric carbon dioxide and water was determined by passing the pretreated air through:-

1) a weighed silica gel absorption tube for 24 hours.

2) a weighed soda lime absorption tube for 24 hours.

The rate of air flow used was 14 litres per hour. In neither case was there an increase in weight greater than 5 mg, showing that water and carbon dioxide had been effectively removed from the air stream.

Temperature of oven and air stream

The temperature of the oven in which the distillation flask was housed was checked over a period of 10 hours and was found to be $100^{\circ} \pm 0.5^{\circ}C$. The temperature of the air stream, over the same

TABLE 2

Recoveries of water after evaporation in oven distillation apparatus

Wt. taken g	Wt. in distillate g	Wt. absorbed by silica gel g	Recovery %
85.00	84.45	0.55	100.00
85.00	84.50	0.47	99.97
85.00	84.56	0.42	99.98
75.00	74.58	0.40	99.97
75.00	74.55	0.45	100.00
75.00	74.52	0.45	99.96

period of time with the oven at the above temperature, was $99.8^{\circ} \pm 0.6^{\circ}C$.

Recovery Tests on Water and Aqueous Solutions of Volatiles

a) Water

The object of this experiment was to determine how much water could be accounted for in the receiving flask and silica gel tube after the evaporation of known weights of water from the distillation flask.

Exactly 85g. of water were weighed into the distillation flask which was immediately transferred to the oven and connected to the rest of the apparatus. Dry, carbon dioxide - free air at $100^{\circ}C$ was passed into the flask at a rate of 14 litres per hour. After 14 hours all the water evaporated from the distillation flask and still head but a few small drops of water remained on the lower end of the condenser. After a further 2 hours this water had been removed from the condenser by the air stream and the apparatus was dismantled. The silica gel and soda lime absorption tubes were weighed at once. But the distillation flask and the receiving flask and delivery tube were stoppered and allowed to return to room temperature before weighing. The receiving flask and delivery tube were weighed together because of the presence of a little ice on the lower end of the delivery tube.

The results of the experiment together with results obtained using 75g. quantities of water are shown in Table 2.

b) Volatile fatty acids

The above experiment was repeated using 85g. quantities of standard solutions of acetic, propionic and butyric acids instead of pure water. The time taken for evaporation was 16 hours and there was again no residue in the distillation flask in any case. After each distillate had been weighed it was titrated with decinormal sodium hydroxide solution using phenolphthalein as indicator. The contents of the weighed silica gel tube were washed with quantities of CO_2 - free water totalling 50 ml. and the washings were titrated with decinormal sodium hydroxide solution. The washings from the still head and condenser were also titrated but were found to be free from acid in every case.

c) Lactic acid

Because lactic acid, even of Analytical Reagent quality, contains small amounts of lactic anydride, it is customary to prepare the pure acid "in situ" by liberation with sulphuric acid from its lithium, calcium or zinc salts. Under the conditions of experiment, however, this procedure was not practicable as it would have resulted in an undesirable concentration of sulphuric acid in the distillation flask in the later stages of evaporation. So 85g. of a solution of lactic acid containing about 1.2g. of the A.R. acid was used. The amount of lactic anhydride in the acid had been previously determined by titrating a sample of the acid before and after hydrolysis with sodium hydroxide. After 18 hours in the apparatus evaporation of water from the distillation flask appeared to be

complete and the apparatus was disconnected and its component parts weighed as before. A residue weighing 0.35g. was found in the distillation flask in the form of a viscous liquid. This residue was only partially soluble in cold water and the sodium hydroxide required for its neutralization to the phenolphthalein end point was equivalent to only about 0.1g. of lactic acid. As it appeared that part of the residue was in the form of lactic anhydride or lactide excess standard sodium hydroxide solution was added to the flask and the mixture warmed for a few minutes to effect reconversion to lactic acid. The volume of alkali used to neutralize the lactic acid formed was determined by a back titration. The percentage of lactic acid remaining non volatile in the oven distillation apparatus was then calculated, allowance being made for the small amount of lactic anhydride originally present.

The amount of volatile lactic acid was determined by titrating the distillate with sodium hydroxide. The washings from the silica gel tube and from the condenser and still head were found to contain no acid.

d) Ammonium hydroxide

Sample of 85g. of a solution of ammonium hydroxide of known concentration were used to test the recovery of ammonia in the apparatus. The distillates and washings from the silica gel tubes were titrated with sulphuric acid solution using methyl red as indicator.

Tests were also carried out with 30 ml. of N acetic acid solution

TABLE 3

Recoveries of pure solutions after evaporation in oven distillation apparatus

	Individual acids, alcohol or ammonium hydroxide				Mixture of acids, alcohol and ammonium hydroxide	
	In distillate %	In silica gel %	In distillate %	In silica gel %	In distillate %	In distillate %
Acetic acid	99.3	0.5	98.8	0.8	98.8	99.6
Propionic acid	99.1	0.6	99.8	0.2	99.2	99.7
Butyric acid	99.3	0.6	98.5	0.6	98.7	99.4
Lactic acid	67.0a	nil	67.7b	nil	69.0c	70.2d
Alcohol	90.8	1.0	91.9	1.5	90.1	91.0
Ammonium hydroxide	90.6	9.0	90.0	9.4	94.7	93.7
Ammonium hydroxide in presence of acetic acid	95.3	4.4	94.8	4.7	-	-

Residual lactic acid after hydrolysis in distillation flask

a 32.7%

b 32.7%

c 30.4%

d 28.3%

initially present in the receiving flask to find out if the recovery of ammonia in the distillate would be improved. In these experiments the ammonia was determined in a Markham micro-Kjeldahl steam distillation apparatus.

d) Alcohol

Samples of 85g. of a solution containing approximately 0.5g. of ethyl alcohol were used. The alcohol content of the original sample, distillates and silica gel washings was determined in suitably diluted portions by Winnick's micro-diffusion technique.

e) Mixture of acids, alcohol and ammonium hydroxide

Recovery tests were carried out using 85g. quantities of a solution containing known amounts of acetic, propionic, butyric and lactic acids, alcohol and ammonium hydroxide. The distillates produced were made up to 200 ml. and aliquot amounts were removed for the determination of the volatile fatty acids, lactic acid, alcohol and ammonia. The washings from the silica gel tubes were not examined.

The recoveries obtained in all the above experiments are shown in Table 3.

Procedure for Determining the Dry Matter and Volatiles in Silage

For the determination of dry matter and volatiles in silage about 100g. of chopped silage were accurately weighed into the distillation flask which was immediately connected to the rest of the apparatus. The temperature of the oven and the air stream was

again 100°C and the rate of air flow used was 14 litres per hour. On completion of the drying process which usually took about 18 hours, the distillation and receiving flasks were stoppered and allowed to return to room temperature before weighing. As in the preliminary experiments the delivery tube was weighed along with the receiving flask. The silica gel and soda lime absorption tubes were also weighed. The contents of the silica gel tube were later washed with water and the washings added to the weighed distillate. The mixture was then made up to 200 ml. with carbon dioxide free water in a graduated flask. The distillates were later analysed for volatile fatty acids, lactic acid, total nitrogen and alcohol.

At the same time as the silage was weighed out for the above determination samples were also weighed for the determination of the volatile fatty acids, lactic acid, total nitrogen, alcohol and pH value. Samples of 100g. silage were also taken for the determination of the apparent dry matter of the silage in a forced draught oven at 100°C, perforated metal trays being used to ensure good air circulation throughout the samples.

RESULTS AND DISCUSSION

The recoveries of water after evaporation in the oven distillation apparatus are shown in Table 2 and it is obvious that within the limits of experimental error all the water was recovered in the receiving flask and silica gel tube. The soda lime absorption tube did not increase in weight in any of the determinations showing that the silica gel was efficient in removing the moisture from the air stream. The recoveries of volatile compounds after evaporation of their aqueous solutions are shown in Table 3 and are, with the exception of alcohol, almost quantitative. Even in the case of alcohol, of which about 7% was unaccounted for, no increase in weight of the soda lime tube was detected. The recoveries of the volatile acids were the same in the presence of alcohol as when examined individually and no esterification therefore could have taken place. A possible explanation is that the alcohol having a lower boiling point (78°C) than water was quickly removed by the hot air stream before it could react with the acids at 100°C.

Silages

Samples of forty-four grass silages ranging in pH value from 3.7 to 5.4 were examined in the oven distillation apparatus. Sixteen of the samples were from the Edinburgh School of Agriculture experimental silo unit and the others were advisory samples obtained from a number of farms in the East of Scotland. All the silages had been made without the use of acid additives or preservatives

TABLE 4

Composition of silages

No	pH	mg/100g. Fresh Silage								per cent		
		Nitrogen		Acetic acid		Butyric acid		Lactic acid		Dry Matter		$\frac{b-a}{b} \times 100$
		Total	Volatile	Total	Volatile	Total	Volatile	Total	Volatile	Apparent a	Corrected b	
1	3.7	586	nil	343	331	nil	nil	1827	156	18.60	19.09	2.55
2	3.7	336	7	373	338	32	30	2004	215	16.32	16.91	3.51
3	3.7	478	9	356	342	8	8	2315	191	20.51	21.07	2.66
4	3.7	397	nil	298	291	nil	nil	1928	187	15.60	16.08	2.99
5	3.8	568	nil	345	316	nil	nil	1660	148	19.29	19.75	2.35
6	3.8	589	nil	358	313	nil	nil	1579	168	18.33	18.81	2.56
7	3.8	346	nil	367	348	80	79	1446	93	16.14	16.67	3.17
8	3.8	326	39	259	217	135	123	1717	104	19.58	20.22[a]	3.17
9	3.8	488	nil	261	255	161	152	1818	145	20.65	21.20	2.59
10	3.8	422	nil	303	285	nil	nil	2046	256	18.75	19.37[b]	3.20
11	3.8	294	10	302	281	20	15	1922	213	19.65	20.27[c]	3.06
12	3.9	571	nil	345	333	nil	nil	1661	146	18.80	19.28	2.48
13	3.9	326	6	368	298	213	206	1418	91	14.70	15.30	3.92
14	3.9	379	5	253	218	24	20	1821	128	15.12	15.49	2.40
15	3.9	374	2	234	186	29	23	1744	168	16.42	16.80	2.25
16	3.9	378	nil	401	375	nil	nil	2021	130	20.81	21.32	2.39
17	4.0	364	2	264	194	29	26	1611	157	16.98	17.34	2.08

TABLE 4 (Contd.)

No	pH	mg/100g. Fresh Silage								per cent		
		Nitrogen		Acetic acid		Butyric acid		Lactic acid		Dry Matter		$\frac{b-a}{b} \times 100$
		Total	Volatile	Total	Volatile	Total	Volatile	Total	Volatile	Apparent a	Corrected b	
18	4.0	330	10	341	320	nil	nil	1321	148	18.38	18.86	2.55
19	4.1	297	8	307	284	336	330	825	54	14.07	14.75	4.61
20	4.1	366	7	266	235	30	23	1736	108	15.97	16.34	2.29
21	4.1	345	12	261	238	23	24	2097	98	18.62	19.07	2.36
22	4.1	310	33	358	328	63	60	2020	199	18.57	19.28[d]	3.68
23	4.2	295	15	361	337	nil	nil	1311	65	16.78	17.20	2.44
24	4.3	669	95	298	257	nil	nil	1989	250	18.22	18.84	3.30
25	4.3	373	44	277	257	67	60	2106	313	35.42	36.13[e]	1.98
26	4.3	292	7	473	368	27	22	684	112	23.08	23.81[f]	3.09
27	4.4	647	35	296	275	nil	nil	1808	216	18.22	18.75	2.84
28	4.5	668	61	397	357	nil	nil	1914	247	16.93	17.61	3.85
29	4.5	455	36	505	490	317	300	1427	110	21.77	22.71	4.14
30	4.6	471	55	419	400	266	243	33	nil	13.81	14.51	4.82
31	4.7	473	31	388	362	380	312	222	3	19.91	20.62	3.47
32	4.7	477	92	315	229	429	424	198	27	23.46	24.35	3.66
33	4.7	405	98	390	364	nil	nil	605	55	21.75	22.27	2.33
34	4.8	229	21	210	168	269	253	1619	11	14.33	14.79	3.08
35	4.9	645	152	319	284	nil	nil	1615	265	17.57	18.30	4.02
36	4.9	427	12	747	589	96	55	2360	47	17.44	18.14	3.89

TABLE 4 (Contd.)

No	pH	mg/100g. Fresh Silage								per cent		
		Nitrogen		Acetic acid		Butyric acid		Lactic acid		Dry Matter		$\frac{b-a}{b} \times 100$
		Total	Volatile	Total	Volatile	Total	Volatile	Total	Volatile	Apparent a	Corrected b	
37	4.9	253	51	661	551	370	299	20	1	15.54	16.45	5.55
38	4.9	268	73	491	460	335	315	299	20	15.62	16.49[g]	5.28
39	5.0	555	91	789	741	610	576	96	10	13.61	15.08	9.75
40	5.0	458	74	576	489	515	498	13	nil	12.80	13.86	7.65
41	5.1	362	133	344	333	661	668	265	11	16.73	17.90	6.56
42	5.2	254	123	555	546	698	629	105	8	13.80	15.13	8.80
43	5.2	635	120	434	389	615	602	19	nil	16.67	17.79[h]	6.30
44	5.4	647	162	649	641	633	607	13	nil	15.82	17.43	9.24

a including 0.15 per cent volatile propionic acid

b including 0.08 per cent volatile alcohol

c including 0.11 per cent volatile alcohol

d including 0.09 per cent volatile propionic acid

e including 0.04 per cent volatile propionic acid

f including 0.23 per cent volatile propionic acid

g including 0.05 per cent volatile propionic acid

h including 0.20 per cent volatile propionic acid

although molasses had been added to several to ensure a good supply of carbohydrate material.

The percentage recoveries (by weight) of the total volatiles in the receiving flask and silica gel tube are given in Table 16 in Appendix I and ranged from 99.60 to 100.34 per cent with a mean recovery of 99.95 per cent. No increase in weight of the soda lime absorption tube was detected and no ammonia was found in the acid wash bottle in any of the determinations.

The apparent dry matter values obtained by weighing the residues in the distillation flasks are shown in Table 17 in Appendix I together with the corresponding values obtained in a forced draught drying oven at 100°C using 100g. samples. The mean difference between the methods was 0.06, significant at a probability rather less than 0.01. This difference is small in comparison with errors due to volatile loss and appears to indicate that the conditions in a forced draught oven have been successfully simulated in the oven distillation apparatus.

The results of the analysis of the distillates and fresh silages are given in Table 4. The apparent dry matter values of the silages have been corrected by adding the total amounts of volatile compounds determined in the distillates. The corrected dry matter values were then used in calculating the amounts of volatile compounds in the fresh silages.

Discussion

The volatile nitrogen expressed as a percentage of the total

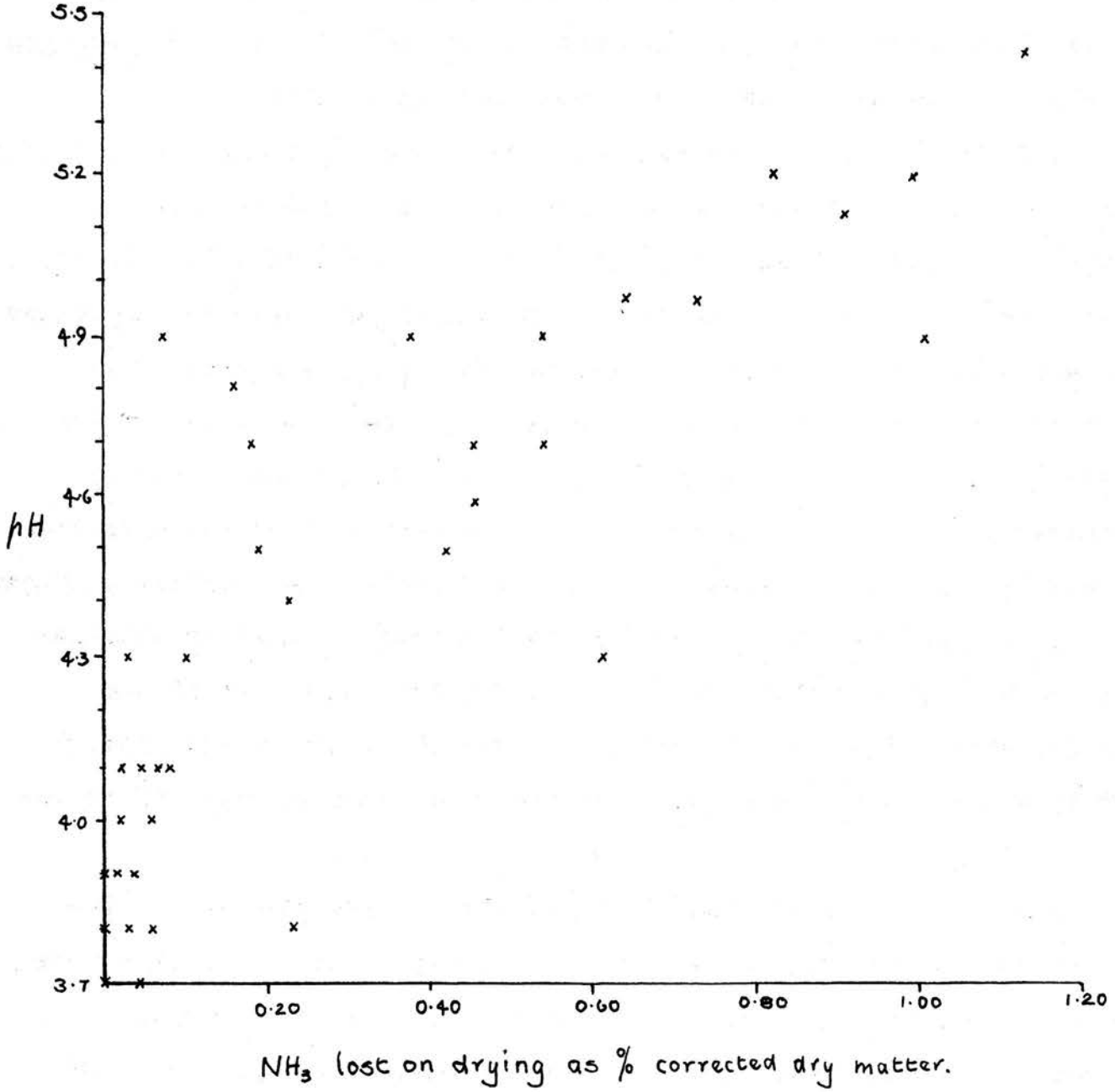

Fig. 4. Variation of NH_3 lost on drying with pH value of silage.

nitrogen in the fresh silage ranged from 0 to 48.4 per cent in the forty four samples examined. The variation of the volatile nitrogen lost on drying, expressed as a percentage of the corrected dry matter, with the pH value of the silage is illustrated graphically in Fig. 4. It is clear that there is an increase in the volatile loss of nitrogen as the pH value increases. This confirms the findings of Virtanen (1934), Watson and Ferguson (1937) and Smith and Comrie (1938). The last-mentioned workers have stated that the critical zone with respect to volatile base production occurs at about pH 4.5. In the present study fifteen of the forty-four samples examined had pH values above 4.5 and in only four of these was the volatile nitrogen less than ten per cent of the total nitrogen; on the other hand, of the twenty-nine silages having pH values of 4.5 or less only three had nitrogen volatilities greater than ten per cent. With the exception of sample 8 all the silages below pH 4 had negligible volatile nitrogen contents. Schoch (1949) has shown that losses of volatile nitrogen can be considerable above pH 5. Unfortunately only four of the silages examined in this investigation had pH values greater than 5 but in all of these the volatile nitrogen was greater than 15% of the total nitrogen.

Acetic acid was present in all of the silages examined. The volatility of the acid ranged from 72.7 to 99.2 per cent with a mean volatility of 90.0 per cent. The amounts of acetic acid present in silage can be considerable and in this investigation thirty-two of the samples examined had acetic acid contents greater than 0.3 per

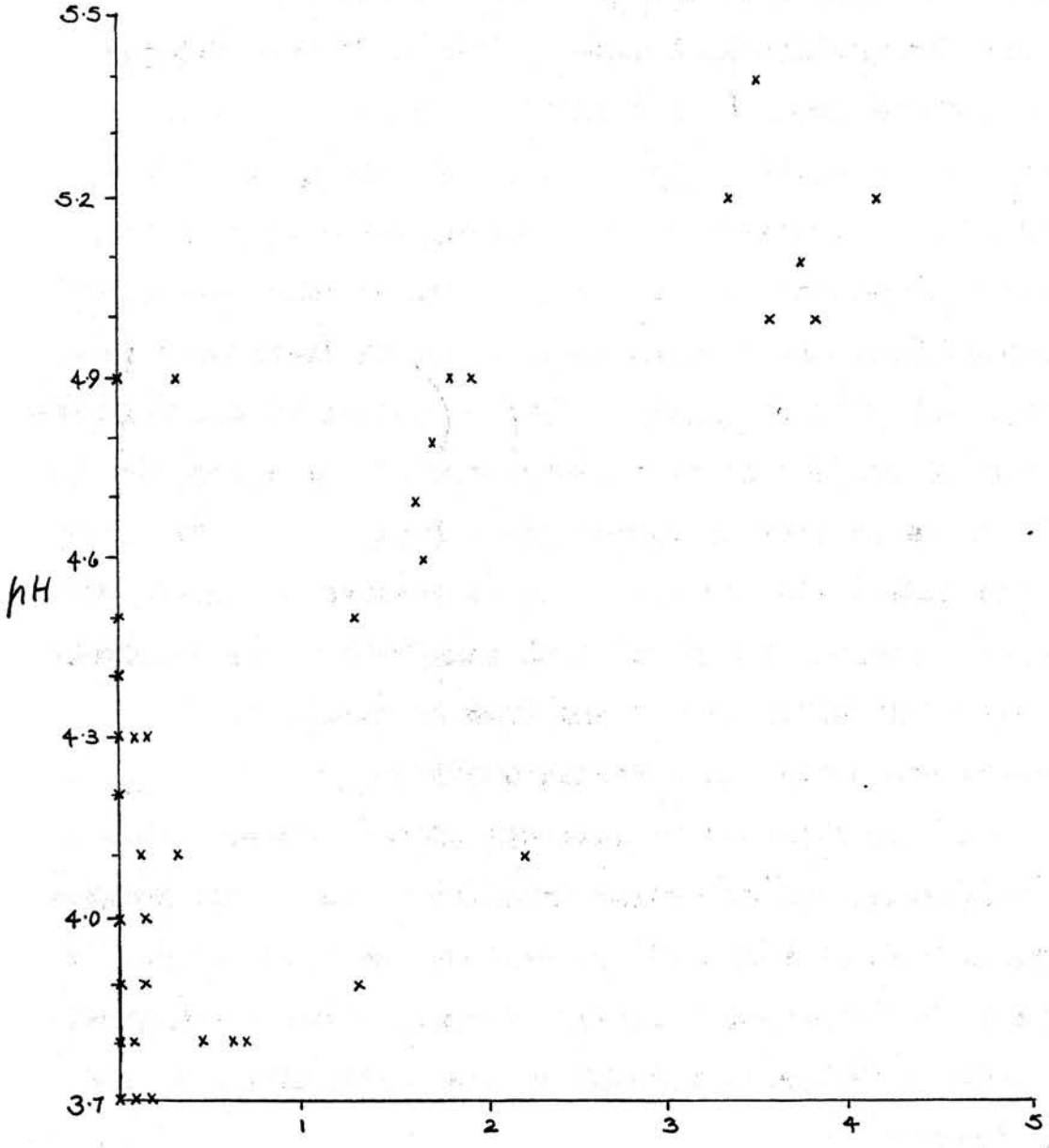

Fig. 5. Variation of butyric acid lost on drying with pH value of silage.

cent of the fresh silage. The acetic acid content was higher in the high pH silages and in several of these was greater than 0.5 per cent. It would appear from a study of the results in Table 4 that acetic acid is the chief volatile constituent of most silages.

Butyric acid was present in thirty of the forty-four silages although in most of the silages with pH values below 4.5 it was present in only small amounts. The percentage volatility of the acid ranged from 57 to 104 per cent with a mean volatility of 90.9 per cent. Some of the higher pH silages contained more than 0.5 per cent of butyric acid in the fresh silage and in seven samples it was the main volatile constituent. The variation of the volatile loss of butyric acid calculated as a percentage of the corrected dry matter, with pH value is shown graphically in Fig. 5. It is clear that the volatile loss due to butyric acid is greater at higher pH values. This association of high pH with high butyric acid content is well known and is a reflection of the type of bacterial fermentation which has taken place during ensilage.

Propionic acid was detected in only six of the silages examined and the percentage volatilities ranged from 118 to 84. The largest quantity of the acid found was 0.23 per cent of the fresh silage in sample 26. Because of the small number of samples which contained this acid it is not possible to comment on its contribution to the volatile error on drying.

Formic acid, which has been found in small amounts in silages by Langston et al (1958), was not present in any of silages analysed

nor were any of the higher volatile fatty acids detected.

It is interesting to note that the mean volatilities of the acetic and butyric acids (90.0 and 90.9 per cent respectively) were considerably higher than the mean volatility of 77 per cent which was determined by Watson and Ferguson (1937) for the total volatile acids. In their experiments, however, a temperature of 98°C was used and the drying time was only 4 hours as compared with 18 hours in this investigation.

Alcohol, which is generally assumed to be a normal constituent of silage, was found in only two samples (nos. 10 and 13). The amounts present were less than the average figure of 0.3% of fresh silage quoted by Watson and Nash (1960). No evidence of the presence of esters was found in either the aqueous extracts of the fresh silage or in the distillates although the amounts of alcohol were so low as to make the results of the analysis unreliable.

Lactic acid was recovered in the distillates from all but four of the silages. The volatility of this acid was also demonstrated in the preliminary experiments with pure solutions although to a much greater extent than was encountered in the silage. The percentage volatility of the lactic acid in the silages ranged from 0.0 to 16.4 with a mean value of 9.3. While these values are lower than those found for the volatile fatty acids they are nevertheless important because lactic acid is usually present in large quantities in well preserved silages. A good illustration of this is seen in sample 10 in which the quantities of lactic acid and

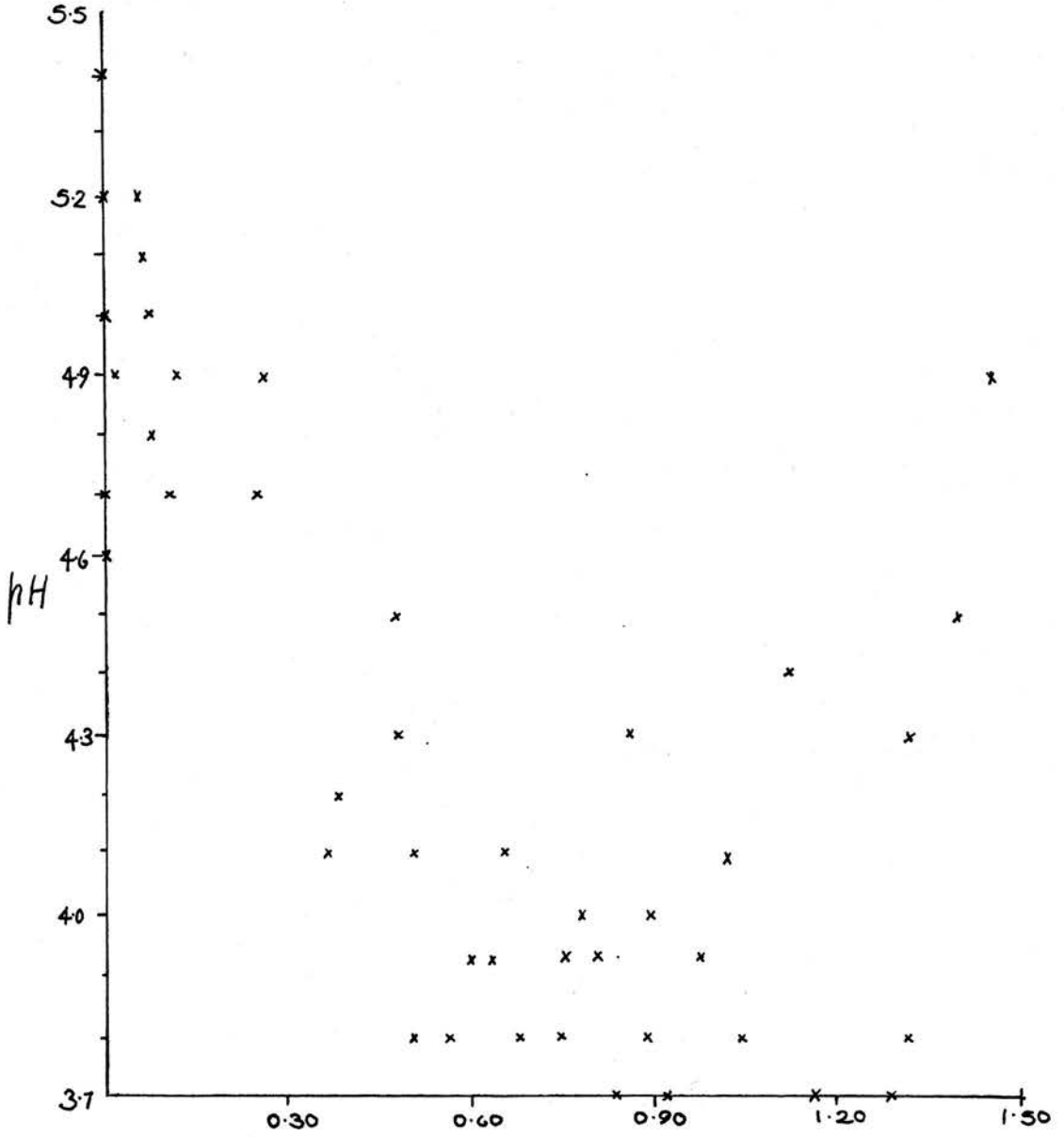

Fig. 6. Variation of lactic acid lost on drying with pH value of silage.

acetic acid lost on drying were 0.256g. and 0.285g. although the respective percentage volatilities were 12.5 and 94.1. The variation of the volatile lactic acid lost on drying with pH is shown in the graph in Fig. 6. In contrast to the loss of ammonia, and the volatile fatty acids the loss of lactic acid decreases with increasing pH because the lactic acid content is lower at high pH values.

Although the steam volatility of lactic acid has been appreciated for a long time there appears to have been no other investigation of the volatility of the acid during the drying of silage. The reason for this may lie in the difficulties in assessing the volatility of the acid by difference calculations due to the formation of lactic anydride and lactide. These changes are represented by the following equations:-

$$2\ CH_3\ CH.OH.COOH \quad = \quad \begin{matrix} CH_3 - CH - O - CO \\ \uparrow \qquad\qquad | \\ CO - O - CH - CH_3 \end{matrix} \quad + \quad 2H_2O$$

Lactic acid — lactide

$$2\ CH_3\ CH.OH.COOH \quad = \quad \begin{matrix} CH_3.\ CH.OH\ CO \\ CH_3.\ CH.OH\ CO \end{matrix} \diagdown O \quad + \quad H_2O$$

Lactic acid — lactic anhydride

In addition another reaction is possible in which lactic acid is converted at 100°C to monolactoyl lactic acid.

$$2\ CH_3.\ CH.OH\ COOH = CH_3\ \underset{\underset{O.CO.CH.OH.CH_3}{|}}{CH.COOH} + H_2O$$

Lactic acid monolactoyl lactic acid

It will be noticed that in the formation of lactic anydride and monolactoyl lactic acid water equivalent to one tenth of the reaction weight of lactic acid is produced, while in the production of lactide the amount of water formed is twice as great. It was therefore of interest to determine to what extent the lactic acid present in silage was involved in these reactions during the drying process as any water produced would lead to a further error in the dry matter determination. Samples of fresh silages of known lactic acid content were therefore dried in the oven distillation apparatus and the amounts of lactic acid lost on drying were determined in the distillates. The dried silage residues were then weighed, mixed and divided into two parts. An aqueous extract of the first part was prepared and the lactic acid content was determined in an aliquot sample in the usual way. The second part was warmed with normal sodium hydroxide solution and the lactic content of an aliquot portion was determined after cooling. A summary of the results, which are given in full in Table 20 in Appendix I, is given below.

Summary of Table 20

Recoveries of lactic acid after drying fresh silage samples in the oven distillation apparatus

	Lactic acid as % lactic acid in fresh silage		
	In distillate %	In aqueous extract of residue %	In alkali extract of residue %
Range	3.2 - 13.4	75.4 - 91.6	85.1 - 97.4
Mean	7.2	82.8	90.4

It can be seen from the table that the mean value for lactic acid recovered in the aqueous extract of the residue is much lower than the corresponding value in the alkali extract. This difference can be explained if it is assumed that some of the lactic acid has been changed at 100°C to a water insoluble compound such as lactic anhydride which will not therefore be extractable from the silage by water; sodium hydroxide, however, will hydrolyse the lactic anydride to lactic acid and a full recovery of the acid might therefore be expected in the alkali extract. The mean percentage of lactic acid which has undergone chemical change is therefore 90.4 - 82.8 or 7.6. In a silage containing 2 per cent of lactic acid the weight of lactic acid changed would be 0.15g. and the greatest possible associated loss of water would be 0.03g.

The total weights of volatile compounds lost during the drying

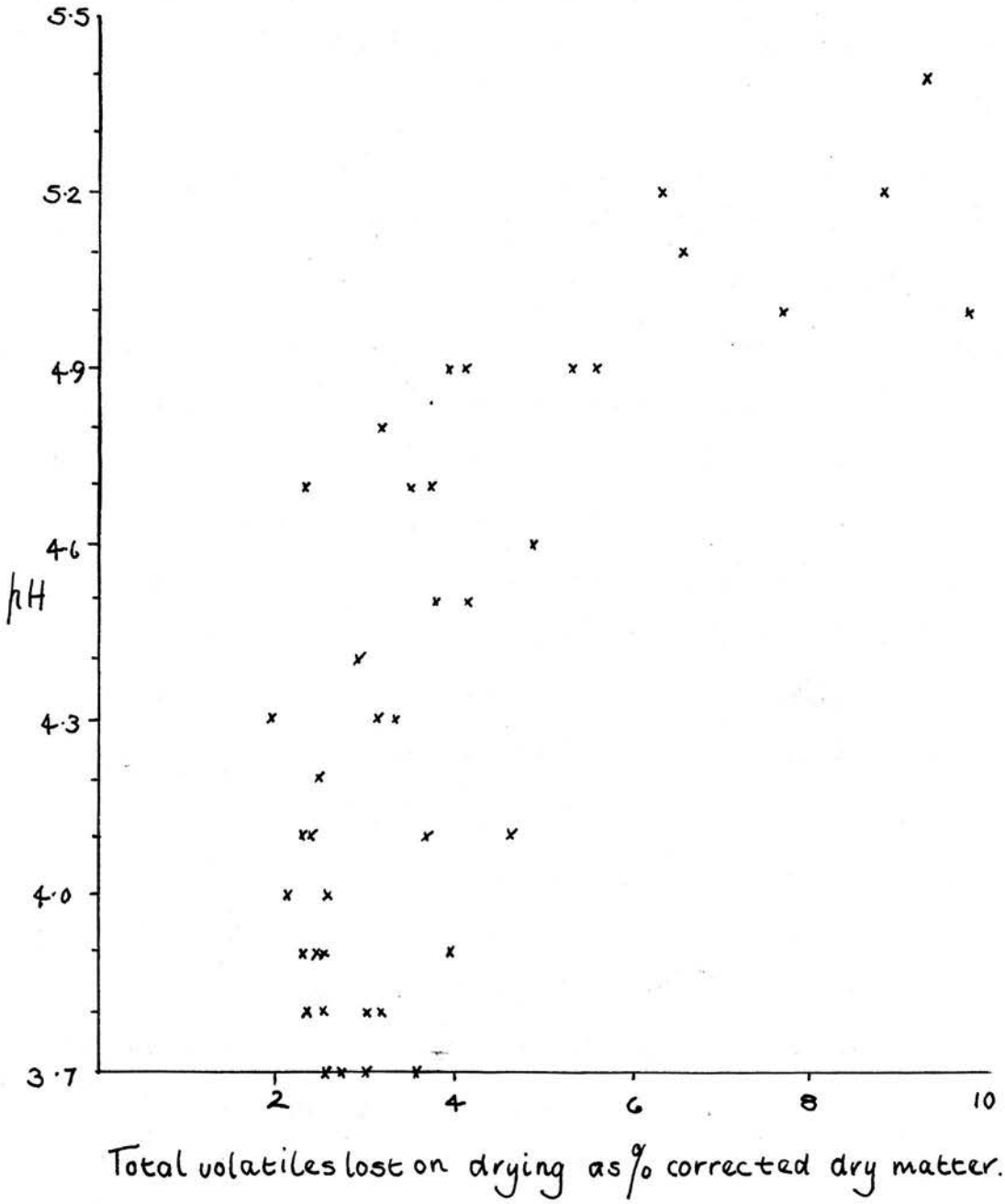

Fig. 7. Variation of total volatiles lost on drying with pH value of silage.

process by the forty-four silages examined are shown in Table 19 as a percentage of the corrected dry matter. In Fig. 7 these values have been plotted against pH value and it is clear that the losses of dry matter are greater at the higher pH values due chiefly to the high amounts of volatile fatty acids and volatile nitrogen present at these pH levels.

As no increase of weight of the soda lime absorption tube was detected in any of the determinations it was inferred that no carbon dioxide had been produced due to dry matter decomposition. It was observed, however, that several of the silages had become slightly brown in colour during drying. Such a change of colour is often regarded as being due to charring and therefore as being indicative of dry matter decomposition. It may, however, be a result of the so-called browning reaction which sometimes takes place on drying foodstuffs. The chemistry of this reaction is not yet fully understood but it has been shown by Maillard (1916) that when solutions of reducing sugars and amino acids are heated together at 100°C a brown pigment is formed. Lewis et al (1949) have found that carboxylic acids such as acetic, propionic, lactic and citric acids may replace the amino acids in the reaction and they have also investigated the production of carbon dioxide during the reaction.

At pH 7.2 which is the optimum pH for the reaction the amount of carbon dioxide produced was only 1 per cent. of the amount of reducing sugar present. The reaction can also take place, though more slowly, at lower pH values and it seems possible that it may

occur in silage which contains an adequate supply of acids and may also contain reducing sugars (McDonald 1960). The amount of carbon dioxide produced should not be very large as the quantities of the reducing sugars are usually less than 1 per cent of the fresh silage.

Although the oven distillation apparatus has been successful in simulating oven drying condition and its use in the determination of silage dry matter is an improvement on existing methods, it is obviously unsuitable as a routine method. It is for this reason that in the following section the toluene distillation method has been examined and the results obtained compared with those in the oven distillation apparatus.

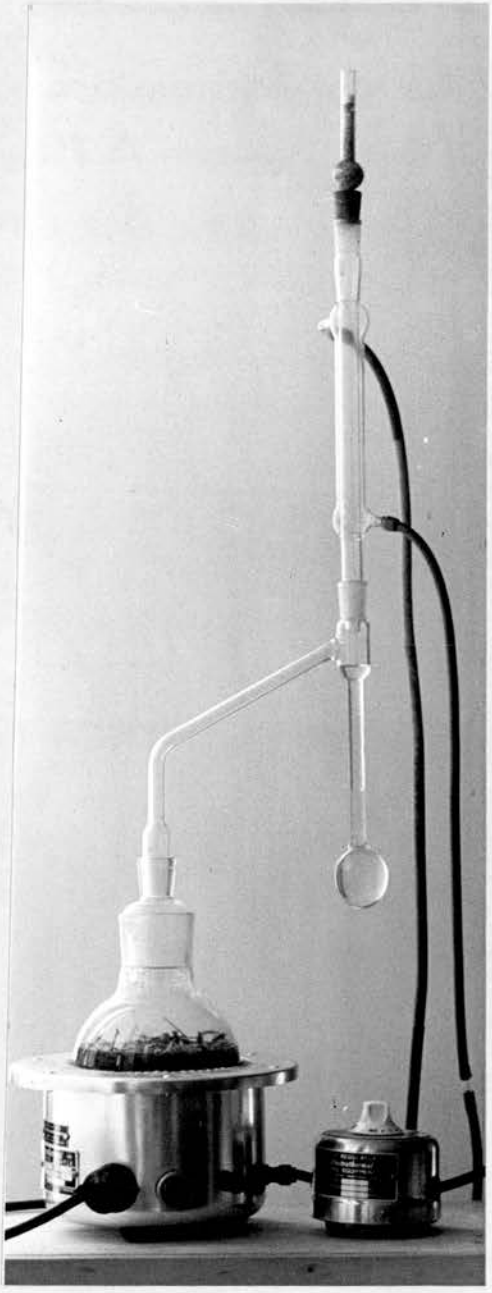

Fig. 8. Toluene distillation apparatus used for the determination of dry matter in silage.

The Determination of Dry Matter by the Toluene Distillation Method

Apparatus

The apparatus used for the determination of dry matter by the toluene distillation procedure is shown in Fig. 8. The trap used was similar to the type designed by Evans and Fetzer (1941) (Fig. 2) and was made by cutting the stem of a conventional small capacity trap and fusing on an ovoid shaped bulb of about 50 ml. capacity. After recalibration the volume scale of the trap was found to be suitable for measuring volumes of water between 51.70 and 58.20 ml. to the nearest 0.05 ml. The trap was connected by means of a reduction adapter to a wide mouthed 1000 ml. round bottomed flask contained in an electro-thermal heating mantle fitted with a fine temperature control. The single surface water condenser used was stoppered with a silica gel absorption tube to exclude interference from atmospheric moisture.

Recovery tests with water

Known volumes of water were distilled with about 400 ml. of toluene until the volume of water collected in the trap became constant. A small amount of water which had collected near the water inlet of the condenser was washed into the trap with a fine jet of toluene added from a long glass tube. Distillation was continued for a further 15 minutes when the washing procedure was repeated. During the distillation the heating mantle was maintained at a temperature which allowed the toluene to drip from the condenser at a rate of about

TABLE 5

Recoveries (by volume) of water in trap after toluene distillation

Volume taken ml.	Volume in trap ml.	Recovery per cent
55.02	55.0	99.96
55.02	55.0	99.96
53.04	53.0	99.92
53.04	53.0	99.92
56.08	56.05	99.94
56.08	56.05	99.94

three drops per second. The total time taken was about four hours. The trap was allowed to stand in a water bath at 20° for about twenty minutes before the volume of the water was read. The percentages of water (by volume) recovered in the trap are shown in Table 5.

Distillation of aqueous solutions of volatile compounds

Samples of aqueous solutions of acetic, propionic, butyric and lactic acids, ammonia and alcohol were examined by the toluene distillation method to find out what proportions of these compounds could be recovered in the aqueous layer of the distillate. The procedure adopted was similar to that used in the previous experiment except that in this case the distillates were retained for analysis. The percentages of volatile compounds recovered in the distillates are shown in Table 6.

Volumes occupied by volatile materials in aqueous solution

Before applying the toluene distillation procedure to silage it was necessary to know the volumes occupied by individual volatile compounds in aqueous solution. These were determined by two methods.

In the first method 100 ml. quantities of the solutions at 20°C were weighed accurately on an analytical balance and the concentrations of the solutions were also determined.

By subtracting the weight of solute present from the weight of solution, the weight of water in the solution was obtained. The volume of water at 20°C was then calculated (density of water at 20°C is 0.99823 per ml.) and by subtracting this from 100 ml. the volume

TABLE 6

Recoveries of volatiles in aqueous distillates from toluene distillation of pure solutions

	Concentration g/100 ml.	Percentage in distillate	Concentration g/100 ml.	Percentage in distillate	Concentration g/100 ml.	Percentage in distillate	Mean percentage in distillate
Acetic acid	0.185	96.8	0.370	97.0	0.740	97.1	96.9
Propionic acid	0.137	65.3	0.274	65.9	0.548	65.0	65.4
Butyric acid	0.181	20.2	0.362	18.5	0.724	19.0	19.2
Lactic acid	0.301	6.5	0.903	7.9	1.505	7.7	7.1
Ammonia	0.038	91.4	0.076	90.8	0.114	90.0	90.5
Alcohol	0.117	85.4	0.234	82.1	0.351	90.0	85.8

occupied by the solute was obtained.

In the second method a 500 ml. graduated flask was filled up to the mark with distilled water at 20°C and 5 ml. of water were removed by pipette. On pipetting 5 ml. of glacial acetic acid into the flask and mixing the contents it was noticed that a small contraction of volume had taken place as the solution no longer came up to the graduation mark. The size of the contraction was measured by running in water from a graduated pipette until the level of the solution returned to the 500 ml. mark. The volume of water in the flask was then 495 ml. plus the volume of water added from the pipette and by subtracting the total volume of water from 500 ml. the volume occupied by the acetic acid was obtained. The weight of 5 ml. of glacial acetic acid at 20°C was also determined in order to calculate the volume occupied by 1g. of the acid in aqueous solution. The volumes occupied by 1g. quantities of propionic, butyric and lactic acids and alcohol were also determined by the same method. The volume occupied by ammonia in aqueous solution could not be measured in this way but the volume occupied by ammonia in acetic acid solution was determined using a slight variation of the method. A solution of acetic acid in water was prepared by adding 5 ml. of glacial acetic acid solution to 495 ml. of water in a 500 ml. graduated flask and making up to the mark with water as before. After withdrawing 5 ml. of the solution 5 ml. of ammonium hydroxide solution were run into the flask. The ammonium hydroxide solution being less dense than the acetic acid solution formed a layer on top of the latter so that at first there was no obvious contraction of volume. On mixing the solutions, however,

TABLE 7

Volumes occupied by 1g. of various volatile compounds in aqueous solution determined by two methods

	conc. of solution g/100 ml.	By Method 1 ml.	By Method 2 ml.	conc. of solution g/100 ml.	By Method 1 ml.	By Method 2 ml.	conc. of solution g/100 ml.	By Method 1 ml.	By Method 2 ml.	Mean volume ml.
Acetic acid	0.2	0.868	0.861	0.6	0.864	0.862	1.0	0.865	0.866	0.864
Propionic acid	0.2	0.918	0.917	0.6	0.914	0.917	1.0	0.916	0.914	0.916
Butyric acid	0.2	0.958	0.961	0.6	0.958	0.954	1.0	0.959	0.957	0.958
Lactic acid	0.2	0.773	0.776	0.6	0.783	0.774	1.0	0.783	0.779	0.778
Alcohol	0.20	1.226	1.219	0.6	1.221	1.215	1.0	1.218	1.215	1.219
Ammonia	0.05	1.598	-	0.1	1.595	-	0.15	1.607	-	1.600
Ammonia (in presence of acid)	0.05	-	0.243	0.1	-	0.246	0.15	-	0.252	0.247

a fairly large contraction took place and this was measured as before. As the concentration and density of the ammonium hydroxide solution were known the volume of water present in 5 ml. of solution was calculated and hence the volume occupied by ammonia in acetic acid solution.

The results obtained by the two different methods are shown in Table 7.

Application of the toluene distillation method to silage

In determining the dry matter of silage 65g. samples of chopped silage were heated with about 400 ml. of toluene. The size of sample used allowed the volumes of distillates from silages with dry matter contents between 11 and 21 per cent to be measured in the trap. Distillates from silages with dry matter contents higher than 21 per cent required the addition of a known volume of water from a pipette before the volume could be read on the volume scale. The time taken for the distillation in most cases varied between seven and eight hours depending on the dry matter content of the silage. After the volumes had been measured, the distillates were made up to 100 ml. with carbon dioxide free water in 100 ml. graduated flasks. The total acid content of each distillate was measured by the method of Foreman (1920) which depends on the fact that in 80 per cent alcoholic solution ammonia is neutral to phenolphthalein. Samples of 20 ml. of the diluted distillates were therefore diluted with 80 ml. of neutral ethanol and titrated with $\frac{N}{10}$ sodium hydroxide solution to the phenolphthalein end point.

Samples of the distillates were also analysed for the individual volatile fatty acids, lactic acid, ammonia and alcohol. Determinations of the volatile compounds present in the fresh silage were also carried out and samples of fresh silage were also examined in the oven distillation apparatus.

RESULTS AND DISCUSSION

The results of the recovery experiments using water (Table 5) show that the toluene distillation apparatus gave almost quantitative recoveries of water.

The quantities of individual volatile compounds recovered in the aqueous distillates after their aqueous solutions had been submitted to the toluene distillation method are shown in Table 6. The results for the volatile fatty acids are of particular interest. The mean recoveries of acetic, propionic and butyric acid were 96.9, 65.4 and 19.2 per cent respectively and it is clear that the volatility of the acids under the conditions of the experiment decreases with increasing molecular weight. These results are at variance with the volatilities of these acids during steam distillation (Duclaux 1874). The comparatively low volatilities of propionic and butyric acids may be explained however, by the fact that the water solubilities of the volatile fatty acids decrease with increasing molecular weight and consequently more of the higher acids are likely to pass into toluene solution in the distillation flask. As the volatile loss of the fatty acids is mainly due to their steam volatility it follows that the acids in toluene solution (i.e. mainly the higher acids) will not be subject to the same loss as those in aqueous solution.

The volatility of lactic acid shown in Table 6 (7 per cent) is in good agreement with the value of 5 per cent found by Smith (1938) during steam distillation. The high proportions of alcohol and ammonia recovered in the distillates are a reflection of the high

water solubility of these substances. The close agreement of results carried out at different concentrations shows, that at the levels examined, concentration has no effect on volatility.

The results obtained by the two methods for determining the volumes occupied by the volatile compounds in aqueous solution are given in Table 7. Again good agreement is shown between results obtained for individual compounds at three concentration levels. There is also close agreement between the results obtained by the two methods used. The volumes occupied by 1g. of acetic, propionic, and butyric acids in aqueous solution are 0.864, 0.916 and 0.958 ml. respectively. The difference between the volumes for the individual acids is due partly to a decrease in the density of the acids with increasing molecular weight (International Critical Tables 1928) and partly to the different contractions undergone by the acids. The difference between the volume occupied by ammonia in water and in acetic acid solution is interesting and is important as acetic acid is usually likely to be present in distillates from silages. The decrease in volume in acid solution is probably due to the formation of ammonium acetate.

Silages

The silages examined by the toluene distillation method were the last sixteen (in chronological order) of the forty-four silages already reported in the previous section on the oven distillation apparatus. The compositions of the sixteen silages including the corrected dry matter values have been abstracted from Table 4 and are

TABLE 8

Composition of silages examined by toluene distillation procedure
(Abstracted from Table 4)

No	pH	mg/100g. Fresh Silage								per cent		
		Nitrogen		Acetic acid		Butyric acid		Lactic acid		Dry Matter		$\frac{b-a}{b} \times 100$
		Total	Volatile	Total	Volatile	Total	Volatile	Total	Volatile	Apparent a	Corrected b	
4	3.7	397	nil	298	291	nil	nil	1928	187	15.60	16.08	2.99
9	3.8	488	nil	261	255	161	152	1818	145	20.65	21.20	2.59
10	3.8	422	nil	303	285	nil	nil	2046	256	18.75	19.37 a	3.20
11	3.8	294	10	302	281	20	15	1922	213	19.65	20.27 b	3.06
16	3.9	378	nil	401	375	nil	nil	2021	130	20.81	21.32	2.39
18	4.0	330	10	341	320	nil	nil	1321	148	18.38	18.86	2.55
22	4.1	310	33	358	328	63	60	2020	199	18.57	19.28 c	3.68
23	4.2	295	15	361	337	nil	nil	1311	65	16.78	17.20	2.44
29	4.5	455	36	505	490	317	300	1427	110	21.77	22.71	4.14
30	4.6	471	55	419	400	266	243	33	nil	13.81	14.51	4.82
33	4.7	405	98	390	364	nil	nil	605	55	21.75	22.27	2.33

TABLE 8 (Contd.)

No	pH	mg/100g. Fresh Silage								per cent		
		Nitrogen		Acetic acid		Butyric acid		Lactic acid		Dry Matter		$\frac{b-a}{b} \times 100$
		Total	Volatile	Total	Volatile	Total	Volatile	Total	Volatile	Apparent a	Corrected b	
38	4.9	268	73	491	460	335	315	299	20	15.62	16.49	5.28
39	5.0	555	91	789	741	610	576	96	10	13.61	15.08 [d]	9.75
40	5.0	458	74	576	489	515	498	13	nil	12.80	13.86	7.65
43	5.2	635	120	434	389	615	602	19	nil	16.67	17.79	6.30
44	5.4	647	162	649	641	633	607	13	nil	15.82	17.43 [e]	9.24

a including 0.08 per cent volatile alcohol

b including 0.11 per cent volatile alcohol

c including 0.09 per cent volatile propionic acid

d including 0.05 per cent volatile propionic acid

e including 0.20 per cent volatile propionic acid

TABLE 9

Volatiles in aqueous distilates obtained by the toluene distillation method using 65g. samples of fresh silage

		mg. in distillate					
No	pH	Ammonia	Acetic acid	Propionic acid	Butyric acid	Lactic acid	Alcohol
4	3.7	nil	177	nil	nil	38	nil
9	3.8	nil	153	nil	10	38	nil
10	3.8	nil	179	nil	nil	31	46
11	3.8	5	171	nil	nil	28	62
16	3.9	nil	234	nil	nil	34	nil
18	4.0	6	188	nil	nil	40	nil
22	4.1	18	204	38	3	27	nil
23	4.2	8	208	nil	nil	29	nil
29	4.5	18	299	nil	12	25	nil
30	4.6	31	251	nil	13	nil	nil
33	4.7	54	227	nil	nil	5	nil
38	4.9	40	279	nil	12	4	nil
39	5.0	51	435	19	27	4	nil
40	5.0	38	282	nil	25	nil	nil
43	5.2	72	229	nil	35	nil	nil
44	5.4	104	345	69	28	nil	nil

TABLE 10

Percentage volatility of acids and nitrogen during the toluene distillation procedure with silages

No	pH	Nitrogen	Acetic acid	Propionic acid	Butyric acid	Lactic acid
4	3.7	-	91.6	-	-	3.0
9	3.8	-	90.2	-	9.6	3.2
10	3.8	-	90.2	-	-	4.2
11	3.8	2.1	87.1	-	-	2.2
16	3.9	-	89.8	-	-	2.6
18	4.0	2.3	87.4	-	-	4.6
22	4.1	7.3	87.7	55.6	7.3	2.1
23	4.2	3.4	91.2	-	-	3.4
29	4.5	5.0	91.1	-	5.8	2.6
30	4.6	8.3	92.2	-	7.5	-
33	4.7	16.9	89.5	-	-	6.2
38	4.9	18.9	87.4	-	5.8	2.1
39	5.0	11.6	84.8	58.5	6.8	6.4
40	5.0	10.5	75.3	-	7.5	-
43	5.2	14.4	85.1	-	8.8	-
44	5.4	20.3	81.7	53.1	6.8	-

TABLE 11

Volumes occupied by volatile constituents in the aqueous distillates from toluene distillation method

Silage Sample	Acetic acid in distillate		Propionic acid in distillate		Butyric acid in distillate		Lactic acid in distillate		Ammonia in distillate		Total Volatiles,
	Wt. a (g)	Volume = a x 0.862 (ml.)	Wt. b (g)	Volume = b x 0.916 (ml.)	Wt. c (g)	Volume = c x 0.958 (ml.)	Wt. d (g)	Volume = d x 0.778 (ml.)	Wt. e (g)	Volume = e x 0.247 (ml.)	Volume (ml.)
4	0.177	0.153	nil	-	nil	-	0.038	0.029	nil	-	0.182
9	0.153	0.132	nil	-	0.010	0.010	0.038	0.030	nil	-	0.172
10	0.179	0.155	nil	-	nil	-	0.031	0.024	nil	-	0.179
11	0.171	0.148	nil	-	nil	-	0.028	0.022	0.005	0.001	0.171
16	0.234	0.202	nil	-	nil	-	0.034	0.026	nil	-	0.228
18	0.188	0.163	nil	-	nil	-	0.040	0.031	0.006	0.002	0.195
22	0.204	0.176	0.038	0.035	0.003	0.003	0.027	0.021	0.018	0.004	0.239
23	0.208	0.180	nil	-	nil	-	0.029	0.023	0.008	0.002	0.205
29	0.299	0.258	nil	-	0.012	0.012	0.025	0.019	0.018	0.004	0.303
30	0.251	0.217	nil	-	0.013	0.013	nil	-	0.031	0.008	0.238
33	0.227	0.196	nil	-	nil	-	0.005	0.004	0.054	0.013	0.200
38	0.279	0.241	nil	-	0.012	0.012	0.004	0.003	0.040	0.010	0.265
39	0.435	0.376	0.019	0.017	0.027	0.026	0.004	0.003	0.051	0.013	0.435
40	0.282	0.244	nil	-	0.025	0.024	nil	-	0.038	0.009	0.277
43	0.229	0.198	nil	-	0.035	0.034	nil	-	0.072	0.018	0.250
44	0.345	0.317	0.069	0.062	0.028	0.027	nil	-	0.104	0.025	0.421

arranged in Table 8 according to their pH values.

The quantities of volatile materials determined in the aqueous distillates from the toluene method are shown in Table 9 and the percentage volatilities of the volatile constituents during the toluene distillation in Table 10. The average percentage volatilities of acetic, propionic, butyric and lactic acids were 87.7, 55.7, 7.3 and 3.6 per cent compared with volatilities of 96.2, 65, 19.2, and 7.1 per cent determined on pure solutions. While lower volatilities might have been expected, because of some of the acids in silage being present in the combined form, it is difficult to explain the large discrepancies in the case of butyric and lactic acids.

In Table 11 the theoretical volumes occupied by the individual volatile constituents in each distillate are shown. These were calculated by multiplying the volume occupied by 1g. of compound in aqueous solution, as shown in Table 7, by the weight of the compound present in the distillate. The total volume occupied by the volatiles in each distillate was also calculated. This volume must be subtracted from the total volume of distillate in order to obtain the volume of water from which the dry matter of the silage can be calculated. Unfortunately, the amount of time taken to analyse the distillate for individual volatile compounds makes the procedure rather long for routine purposes. An attempt was therefore made to relate the volume occupied by the total acids and ammonia in the distillate to the volume of $\frac{N}{10}$ alkali solution equivalent to the total acids in the distillate. This latter volume can be determined quickly, without

TABLE 12

ml. of $\frac{N}{10}$ alkali equivalent to weights of individual acids present in the aqueous distillates from toluene distillation

No	Acetic acid	Propionic acid	Butyric acid	Lactic acid	Total acids
4	29.5	-	-	4.2	33.7
9	25.5	-	1.1	4.2	30.8
10	29.8	-	-	3.5	33.3
11	28.5	-	-	3.1	31.6
16	39.0	-	-	3.7	42.7
18	31.2	-	-	4.4	35.7
22	34.0	5.0	0.3	3.0	42.3
23	34.7	-	-	3.2	37.9
29	49.8	-	1.4	2.8	54.0
30	41.8	-	1.5	-	43.3
33	37.8	-	-	0.6	38.4
38	46.5	-	1.4	0.4	48.3
39	72.6	2.6	3.0	0.4	78.6
40	47.0	-	2.8	-	49.8
43	38.2	-	4.0	-	42.2
44	57.6	9.3	3.1	-	70.0

TABLE 13

Calculation of factor F for converting ml. $\frac{N}{10}$ alkali to volume occupied by acids and ammonia

No	Volume (ml.) occupied by acids and ammonia in distillate a	ml. $\frac{N}{10}$ alkali equivalent to acids in distillate b	$\frac{a}{b}$ (F)
4	0.182	33.7	0.00540
9	0.172	30.8	0.00558
10	0.179	33.3	0.00538
11	0.171	31.6	0.00541
16	0.228	42.7	0.00540
18	0.195	35.7	0.00585
22	0.239	42.3	0.00565
23	0.205	37.9	0.00544
29	0.303	54.0	0.00561
30	0.238	43.3	0.00550
33	0.200	38.4	0.00521
38	0.265	48.3	0.00549
39	0.435	78.6	0.00554
40	0.277	49.8	0.00556
43	0.250	42.2	0.00569
44	0.421	70.0	0.00615

TABLE 14

Calculation of the corrected dry matter value by the toluene distillation method using the Factor F

No	Observed volume of distillate at 20°C ml.	Apparent dry matter %	ml. $\frac{N}{10}$ alkali required in Foreman titration t	Volume to be subtracted from observed volume of distillate t x F(0.00555)	Corrected volume of distillate ml.	Corrected dry matter %
4	54.75	15.88	33.65	0.187	54.56	16.21
9	51.55	20.84	28.65	0.159	51.39	21.07
10	52.80	18.92	34.00	0.189	52.61	19.20
11	52.15	19.93	31.65	0.171	51.98	20.20
16	51.45	21.00	42.55	0.236	51.21	21.37
18	53.05	18.53	35.95	0.200	52.85	18.84
22	52.70	19.07	42.05	0.233	52.47	19.42
23	54.10	16.92	37.60	0.209	53.89	17.23
29	50.60	22.31	54.50	0.303	50.30	22.77
30	55.90	14.16	43.30	0.240	55.66	14.51
33	50.90	21.84	38.40	0.213	50.69	22.16
38	54.80	15.84	48.65	0.270	54.53	16.27
39	55.70	14.47	77.80	0.432	55.27	15.13
40	56.35	13.46	49.55	0.275	56.08	13.96
43	54.85	17.31	42.15	0.234	53.52	17.81
44	54.15	16.88	71.05	0.395	53.65	17.46

interference from ammonia, by the titration method of Foreman. The volumes of $\frac{N}{10}$ alkali equivalent to the individual acids in the distillate were calculated (Table 12) and hence the volume equivalent to the total acids. This volume of alkali was used in Table 13 in the calculation of a titration factor F which is

$$\frac{\text{the total volume occupied by acids and ammonia}}{\text{volume of } \frac{N}{10} \text{ alkali equivalent to the total acids.}}$$

The mean value of F thus obtained was 0.00555 and the standard deviation was 0.0003. If the volume of $\frac{N}{10}$ alkali equivalent to the total acids is known from a titration, multiplication by the factor F should give the approximate volume due to acids and ammonia in the distillate.

In Table 14 the observed volumes of the distillates from the sixteen silages examined are shown together with the calculated dry matter assuming that the distillates consisted only of water. In making these and subsequent calculations the volume of water was multiplied by its density at 20°C (0.99823 g/ml.) in order to convert it to a weight. The volumes of $\frac{N}{10}$ alkali required in the Foreman titrations are also shown in Table 14 and are in good agreement with the theoretical volumes of alkali equivalent to the total acids (Table 12). The Foreman titres were therefore multiplied by the titration factor F to get approximate figures for the volumes of total acids and ammonia in the distillate. In each case the volumes obtained have been subtracted from the observed volumes of the distillates and the dry matter values based on the corrected volumes

TABLE 15

Comparison of percentage dry matter values obtained in oven distillation method and in toluene distillation method

pH	Oven distillation apparatus at 100°C				Toluene distillation procedure			
	No	Uncorrected a	Corrected b	$\frac{b-a}{b} \times 100$	Uncorrected c	$\frac{b-c}{b} \times 100$	Corrected d	$\frac{b-d}{b} \times 100$
3.7	4	15.60	16.28	2.99	15.88	1.24	16.21	- 0.81
3.8	9	20.65	21.20	2.59	20.84	1.70	21.07	0.61
3.8	10	18.75	19.37	3.20	18.92	2.32	19.20	0.87
3.8	11	19.65	20.27	3.06	19.93	1.68	20.20	0.35
3.9	16	20.81	21.32	2.39	21.00	1.50	21.37	- 0.24
4.0	18	18.38	18.86	2.55	18.53	1.75	18.84	0.16
4.1	22	18.57	19.28	3.68	19.07	1.09	19.42	- 0.73
4.2	23	16.78	17.20	2.44	16.92	1.63	17.23	- 0.17
4.5	29	21.77	22.71	4.14	22.31	1.76	22.77	- 0.26
4.6	30	13.81	14.51	4.82	14.16	2.25	14.51	$\pm$ 0.00
4.7	33	21.75	22.27	2.33	21.84	1.93	22.16	0.49
4.9	38	15.62	16.49	5.28	15.84	3.94	16.27	1.34
5.0	39	13.61	15.08	9.75	14.47	4.04	15.13	- 0.33
5.0	40	12.80	13.86	7.65	13.46	2.89	13.96	- 0.72
5.2	43	16.67	17.79	6.30	17.31	2.70	17.81	- 0.10
5.4	44	15.82	17.43	9.24	16.88	3.15	17.46	- 0.20

have been calculated.

The dry matter values, corrected and uncorrected, obtained in the oven distillation apparatus and in the toluene distillation procedure are compared in Table 15.

The results obtained by the uncorrected toluene method are in every case lower than those obtained by the corrected oven distillation method. The mean difference between the results of the two methods is 0.41 per cent which is highly significant at a probability of 0.01. The presence of acetic acid, which was the main volatile constituent of every distillate, may be regarded as the main cause of the low results in the toluene method. The differences between the results are slightly greater at high pH values but the increase is not so marked as in the differences between the corrected and uncorrected results in the oven distillation apparatus chiefly because of the low volatility of butyric acid in the toluene method.

The corrected dry matter values obtained by the toluene method are in good agreement with the corrected results of the oven distillation method. The mean difference between the two sets of results is 0.02 per cent which is significant at a probability rather less than 0.5. The factor of 0.00555 which was used in converting the Foreman titre to the total volume occupied by volatiles in the distillate is slightly higher than the factor 0.00518 which would be used if only acetic acid was present, and allowance is therefore made for the presence of other acids. The corresponding factors for propionic, butyric and lactic acids are 0.00678, 0.00843 and 0.0700 respectively. The greatest error

likely to result from the use of 0.00555 as a factor would occur if butyric acid was the only volatile acid in the distillate when the butyric acid would be underestimated by $\frac{0.00843 - 0.00555}{0.00843} \times 100$ or 34 per cent. As only a small proportion of the butyric acid in silage appears to be volatile during the toluene distillation procedure, the error involved is likely to be fairly small. Ammonia would cause serious interference only if present in a fairly large excess. A study of Table 4, however, shows that at high pH values, at which the greatest loss of ammonia takes place, there is usually enough volatile acetic acid present in the silages to neutralize the ammonia given off.

The presence of alcohol in the distillate could cause an error additional to that calculated by means of the titration factor. In silages 10 and 11, failure to take the volume due to alcohol into account has resulted in the dry matter values being underestimated by about 0.5 and 0.75% of the corrected dry matter.

The fact that higher results are obtained by the uncorrected toluene method than by the uncorrected oven drying method agrees with the observations of Perkins (1943) and Wittwer (1958). The results in the present investigation, however, show that these workers have been wrong in neglecting to allow for the volume of volatile compounds in the distillates.

In conclusion it may be said that the best method of obtaining an accurate figure for silage dry matter is by the oven distillation procedure. However, the method is time consuming and because of the use of the oven only one silage sample may be examined at a time.

On the other hand, the toluene distillation method is completed within eight hours and several determinations may be carried out simultaneously. If the total acids in the aqueous distillate are titrated by the Foreman method and the correction factor F is applied the results should be reasonably close to those obtained by the oven distillation method.

SUMMARY

1. An apparatus was designed in which the water and volatiles lost on drying silage at 100°C could be collected quantitatively for analysis. Forty-four silages, ranging in pH value from 3.7 to 5.4 were examined in the apparatus and the distillates produced were analysed for volatile fatty acids, lactic acid, ammonia and alcohol. In each case the total volatile loss was added to the apparent dry matter value to obtain a value for corrected dry matter.

Acetic acid was the main volatile compound found in the silages and its mean volatility was found to be 90.0 per cent. Appreciable volatile losses of butyric acid and ammonia occurred in silages with high pH values. Lactic acid was also found in the silage distillates and had a mean volatility of 9.3 per cent. Total losses of volatiles ranging from 1.98 to 9.75 per cent of the corrected dry matter were found.

2. An improved method for the determination of silage dry matter by the toluene distillation procedure is described. By titrating the total acids present in the aqueous distillate with $\frac{N}{10}$ alkali and multiplying by a Factor F an approximate value for the volume of volatiles in the distillate may be obtained. Dry matter values for silages determined by this method show good agreement with those obtained by the oven distillation apparatus.

APPENDIX I

TABLE 16

Percentage recoveries (by weight) in oven distillation apparatus

No	In residue	In distillate	In silica gel tube	Total recovery	No	In residue	In distillate	In silica gel tube	Total recovery
1	18.60	80.76	0.59	99.95	23	16.78	82.53	0.70	100.01
2	16.32	82.94	0.70	99.96	24	18.22	81.39	0.63	100.24
3	20.51	78.82	0.65	99.98	25	35.42	63.98	0.48	99.88
4	15.60	83.74	0.71	100.05	26	23.08	76.20	0.65	99.93
5	19.29	80.10	0.65	100.04	27	18.22	80.90	0.72	99.84
6	18.33	80.99	0.65	99.97	28	16.93	82.02	0.75	99.70
7	16.14	83.05	0.51	99.70	29	21.77	77.37	0.81	99.95
8	19.58	79.45	0.73	99.76	30	13.81	85.62	0.54	99.97
9	20.65	78.57	0.73	99.95	31	19.91	79.45	0.54	99.90
10	18.75	80.59	0.65	99.99	32	23.46	76.11	0.77	100.34
11	19.65	79.57	0.69	99.91	33	21.75	77.51	0.71	99.97
12	18.80	80.59	0.58	99.97	34	14.33	84.84	0.81	99.98
13	14.70	84.36	0.79	99.85	35	17.57	81.66	0.66	99.89
14	15.12	83.84	0.64	99.60	36	17.44	81.68	0.76	99.88
15	16.42	82.74	0.80	99.96	37	15.54	83.92	0.51	99.97
16	20.81	78.51	0.61	99.93	38	15.62	83.59	0.69	99.90
17	16.98	82.54	0.71	100.23	39	13.61	85.67	0.70	99.98
18	18.38	80.99	0.55	99.92	40	12.80	86.41	0.73	99.94
19	14.07	85.18	0.66	99.91	41	16.73	82.53	0.69	99.95
20	15.97	83.25	0.69	99.91	42	13.80	85.40	0.60	99.80
21	18.62	80.24	0.54	100.00	43	16.67	82.71	0.64	100.02
22	18.57	80.57	0.84	99.98	44	15.82	83.60	0.59	100.01

TABLE 17

Apparent percentage dry matter value of silages dried in oven distillation apparatus and forced draught oven at 100°C

No	In oven distillation apparatus A	In forced draught oven at 100°C B	A - B	No	In oven distillation apparatus A	In forced draught oven at 100°C B	A - B
1	18.60	18.47	+ 0.13	23	16.78	16.65	+ 0.13
2	16.32	16.50	- 0.18	24	18.22	18.15	+ 0.07
3	20.51	20.42	+ 0.09	25	35.42	35.27	+ 0.15
4	15.60	15.48	+ 0.12	26	23.08	22.91	+ 0.17
5	19.29	19.44	- 0.15	27	18.22	18.03	+ 0.19
6	18.33	18.20	+ 0.13	28	16.93	16.85	+ 0.08
7	16.14	16.21	- 0.07	29	21.77	21.74	+ 0.03
8	19.58	19.43	+ 0.15	30	13.81	13.88	- 0.07
9	20.65	20.48	+ 0.17	31	19.91	19.86	+ 0.05
10	18.75	18.79	+ 0.04	32	23.46	23.26	+ 0.20
11	19.65	19.54	+ 0.11	33	21.75	21.90	- 0.15
12	18.80	18.80	± 0.00	34	14.33	14.31	+ 0.02
13	14.70	14.62	+ 0.08	35	17.57	17.37	+ 0.20
14	15.12	15.15	- 0.03	36	17.44	17.31	+ 0.13
15	16.42	16.66	- 0.24	37	15.54	15.50	+ 0.04
16	20.81	20.88	- 0.07	38	15.62	15.39	+ 0.23
17	16.98	16.87	- 0.11	39	13.61	13.60	+ 0.01
18	18.38	18.39	- 0.01	40	12.80	12.75	+ 0.05
19	14.07	18.89	+ 0.18	41	16.73	16.58	+ 0.15
20	15.97	15.80	+ 0.17	42	13.80	13.92	- 0.12
21	18.62	18.54	+ 0.08	43	16.67	16.74	- 0.07
22	18.57	18.49	+ 0.08	44	15.82	15.70	+ 0.12

Mean difference = + 0.06 Standard deviation of differences = 0.11

Standard error of difference = .017 t = 3.52

TABLE 18

Percentage volatility of silage constituents during drying in oven distillation apparatus

No	pH	Nitrogen (as % total N)	Acetic acid	Propionic acid	Butyric acid	Lactic acid
1	3.7	-	96.5	-	-	8.5
2	3.7	2.1	90.6	-	93.8	10.7
3	3.7	1.9	96.1	-	100.0	8.3
4	3.7	-	97.6	-	-	9.7
5	3.8	-	91.6	-	-	8.9
6	3.8	-	87.4	-	-	10.6
7	3.8	-	94.8	-	98.8	6.4
8	3.8	11.9	83.8	117.6	91.1	6.1
9	3.8	-	97.8	-	94.4	7.9
10	3.8	-	94.1	-	-	12.5
11	3.8	2.8	94.0	-	75.0	11.1
12	3.9	-	96.5	-	-	8.8
13	3.9	1.8	81.0	-	96.7	6.4
14	3.9	1.3	86.2	-	83.3	7.0
15	3.9	0.5	79.5	-	79.3	9.6
16	3.9	-	93.1	-	-	6.4
17	4.0	0.5	73.5	-	89.7	9.7
18	4.0	2.5	93.5	-	-	11.2
19	4.1	2.7	92.5	-	98.2	6.5
20	4.1	1.9	88.3	-	76.7	6.2
21	4.1	3.5	91.2	-	104.3	4.7

TABLE 18 (Contd.)

No	pH	Nitrogen (as % total N)	Acetic acid	Propionic acid	Butyric acid	Lactic acid
22	4.1	4.1	95.8	83.8	95.3	9.9
23	4.2	4.2	91.6	-	-	4.9
24	4.3	14.2	86.2	-	-	12.6
25	4.3	11.8	92.8	92.1	89.6	14.9
26	4.3	2.4	77.8	85.6	81.5	16.4
27	4.4	5.4	92.9	-	-	11.9
28	4.5	9.1	89.9	-	-	12.9
29	4.5	6.5	93.3	-	94.6	7.7
30	4.6	9.6	97.0	-	91.2	-
31	4.7	6.6	93.3	-	82.1	1.4
32	4.7	19.3	72.7	-	98.8	13.6
33	4.7	19.9	95.5	-	-	9.9
34	4.8	9.2	80.0	-	94.1	6.8
35	4.9	23.6	89.0	-	-	16.4
36	4.9	2.8	78.8	-	57.3	2.0
37	4.9	20.2	83.4	-	80.8	5.0
38	4.9	22.4	93.3	-	94.0	6.7
39	5.0	13.5	93.9	100.0	94.4	10.4
40	5.0	13.3	84.9	-	97.1	-
41	5.1	36.7	96.8	-	101.1	4.2
42	5.2	48.4	98.4	-	90.1	7.6
43	5.2	15.6	89.6	-	97.9	-
44	5.4	20.6	99.0	95.2	95.9	-

TABLE 19

Volatile constituents lost on drying as percentage of the corrected dry matter of silage

No	pH	Ammonia	Acetic acid	Propionic acid	Butyric acid	Lactic acid	Total volatiles
1	3.7	-	1.73	-	-	0.82	2.55
2	3.7	0.05	2.00	-	0.18	1.29	3.51
3	3.7	0.05	1.62	-	0.04	0.91	2.66
4	3.7	-	1.81	-	-	1.16	2.99
5	3.8	-	1.60	-	-	0.75	2.35
6	3.8	-	1.66	-	-	0.89	2.56
7	3.8	0.03	2.10	-	0.47	0.56	3.17
8	3.8	0.23	1.07	0.73	0.61	0.51	3.17
9	3.8	-	1.20	-	0.72	0.68	2.59
10	3.8	-	1.47	-	-	1.32	3.20
11	3.8	0.06	1.39	-	0.07	1.05	3.06
12	3.9	-	1.72	-	-	0.76	2.48
13	3.9	0.04	1.95	-	1.31	0.63	3.92
14	3.9	0.04	1.41	-	0.13	0.08	2.40
15	3.9	0.01	3.11	-	0.14	0.99	2.25
16	3.9	-	1.76	-	-	0.61	2.39
17	4.0	0.02	1.20	-	0.15	0.90	2.05
18	4.0	0.06	1.70	-	-	0.78	2.55
19	4.1	0.07	1.93	-	2.24	0.37	4.61
20	4.1	0.05	1.44	-	0.14	0.66	2.29
21	4.1	0.08	1.75	-	0.13	0.51	2.36

TABLE 19 (Contd.)

No	pH	Ammonia	Acetic acid	Propionic acid	Butyric acid	Lactic acid	Total volatiles
22	4.1	0.21	1.70	0.46	0.31	1.03	3.68
23	4.2	0.01	1.90	-	-	0.38	2.44
24	4.3	0.61	1.37	-	-	1.32	3.30
25	4.3	0.15	0.71	1.10	0.17	0.87	1.98
26	4.3	0.03	1.55	0.95	0.09	0.48	3.09
27	4.4	0.23	1.47	-	-	1.15	2.84
28	4.5	0.42	2.03	-	-	1.40	3.85
29	4.5	0.19	2.16	-	1.32	0.48	4.14
30	4.6	0.46	2.75	-	1.67	-	4.82
31	4.7	0.18	1.76	-	1.52	0.01	3.47
32	4.7	0.46	0.94	-	1.74	0.11	3.66
33	4.7	0.54	1.63	-	-	0.25	2.33
34	4.8	0.19	1.13	-	1.71	0.08	3.08
35	4.9	0.01	1.55	-	-	1.45	4.02
36	4.9	0.08	3.24	-	0.31	0.26	3.89
37	4.9	0.38	3.35	-	1.82	0.01	5.55
38	4.9	0.54	2.79	-	1.91	0.12	5.28
39	5.0	0.73	4.92	0.33	3.82	0.07	9.75
40	5.0	0.65	3.53	-	3.59	-	7.65
41	5.1	0.91	1.86	-	3.73	0.06	6.56
42	5.2	0.99	3.61	-	4.16	0.05	8.80
43	5.2	0.82	2.14	-	3.38	-	6.30
44	5.4	1.13	3.68	1.15	3.48	-	9.24

TABLE 20

Recoveries of lactic acid after drying fresh silages in the oven distillation apparatus

No	pH	Lactic acid in 100g. fresh silage A g	Lactic acid in distillate		Lactic acid in aqueous extract		Lactic acid in alkali extract	
			g	as %age of A	g	as %age of A	g	as %age of A
45	3.7	3.29	0.11	3.2	2.87	87.3	3.02	91.9
46	3.9	2.44	0.13	5.2	1.98	80.9	2.26	92.7
47	3.9	2.04	0.22	10.5	1.54	75.4	1.78	88.1
48	4.0	1.54	0.15	9.6	1.23	79.7	1.37	89.0
49	4.0	1.81	0.25	13.4	1.51	80.7	1.60	85.3
50	4.2	1.69	0.13	7.5	1.46	86.5	1.55	92.0
51	4.3	1.75	0.10	5.4	1.38	79.2	1.45	85.1
52	4.4	1.11	0.04	3.4	1.01	91.6	1.08	97.4
53	4.5	0.41	0.03	6.1	0.35	84.3	0.37	90.3
54	4.6	0.64	0.05	7.8	0.53	82.1	0.59	91.9

APPENDIX II

Determination of the Volatile Fatty Acids

Apparatus

Chromatographic tubes with fritted-glass filter and delivery stopcock. Inside diameter of tube 18 mm. Length (top to stopcock) 5 cm.

Reagents

(i) Celite:- Johns Manvilles' Analytical Filter Aid.

(ii) Petroleum Ether (b.p. 63 - 70°):- prepared by distilling A.R. petroleum ether (b.p. 60 - 80°C) from a flask containing sodium hydroxide pellets and collecting the fraction distilling between 63° and 70°C.

(iii) Acetone A.R.:- redistilled.

(iv) Cresol Red Indicator:- 13 ml. of $\frac{N}{10}$ NaOH are added to 0.05g. of o-cresolsulphonaphthalein in 20 ml. of ethanol and the volume made up to 50 ml. with water.

(v) Alphamine Red R Indicator:- solution of 0.5g. in 100 ml. of water.

Preparation of adsorbent

12 ml. of alphamine - red - R indicator solution are mixed with 30 ml. of sucrose solution (2 parts sugar to 1 part water) and 0.5 ml. $\frac{N}{10}$ H_2SO4 added. The mixture is then added slowly to a swirling suspension of 75g. Celite in 750 ml. of petroleum ether and acetone

mixture (1:1 $\frac{v}{v}$) in a homogenizer and vigorous stirring is continued for three minutes. Adsorbent thus prepared may be stored in glass stoppered flasks in a refrigerator.

Developing Solvents

Mixtures containing the following percentages by volume of acetone in purified petroleum ether are prepared:- 1, 5, 10, 15, 20 and 30%. These are referred to as PA_1, PA_5 etc. Concentrations above PA_5 are equilibrated against the static phase by shaking 4 litres of solvent with 100 ml. of 50% sucrose solution to which has been added 2 ml. of saturated barium hydroxide solution and a few drops of cresol red indicator solution to free the solvent of carbon dioxide and traces of acids; after settling the solvent is freed of suspended droplets by passing through filter paper.

Column preparation

A chromatographic tube is filled to within one inch of the top with adsorbent and a tamping rod is passed lightly down the tube to remove air bubbles. The adsorbent is compressed to a fixed volume by air pressure and about 20 ml. of PA_1 added. Cap material consisting of 8g. of sodium sulphate, Celite and ammonium sulphate in the proportions of 12:8:1 is added in the form of a slurry in 25 ml. of PA_1. After compression of the cap material about 75 ml. of PA_1 are forced through the column to remove the PA_{50} initially present and the supernatant liquid expressed to the level of cap material.

Sample introduction

A cavity extending $\frac{2}{3}$ down the centre of the cap material is made with a glass rod and enlarged with an off-centre motion of the rod. A 2 ml. sample of acids in aqueous sulphuric acid solution is pipetted into the cavity and 25 ml. of PA_1 added. The cap material is then homogenized, by means of a specially shaped metal rod held in an electric stirrer, and compressed to a height of 6 cm.

Column development

Pressure is adjusted to give a solvent flow rate of 2 - 3 drops per second. PA_1 elutes butyric and higher acids and the progress of the acids down the column may be followed by the indicator change from orange to blue. PA_5, PA_{10} and PA_{15} may be used individually to remove propionic and acetic acids in order. Lower concentrations of acetone give wider separations; higher concentrations give faster elutions in smaller volumes. Receiver flasks are changed when the blue zone comes to within 1 cm. of the filter plate.

Titration of acids

Titrations of the eluted acids are carried out, in the presence of 50 ml. carbon dioxide free water, against $\frac{N}{200}$ alkali solution using cresol red as indicator. Atmospheric carbon dioxide is excluded by bubbling carbon dioxide free air through the flask during the titration.

Preparation of silage extract

Samples of 50g. finely chopped silage are introduced into 4 oz. bottles with plastic screw caps. The sample is covered with 50ml. 0.6N

TABLE 21

Recoveries of known quantities of volatile fatty acids added to a silage extract

	Butyric acid	Propionic acid	Acetic acid	Formic acid
Wt. in silage extract (g)	0.151	nil	0.251	nil
Wt. of acid added	0.244	0.211	0.310	245
Wt. of acid found in mixture	0.390	0.207	0.554	0.240
No. of determinations	6	6	6	6
Mean wt. of acid recovered	0.239	0.207	0.303	0.240
Mean percentage recovery	97.92	98.10	98.38	97.95

sulphuric acid solution and stored in a refrigerator for one week. The contents are then mixed and compressed with a flat headed glass rod so that the liquid can be drained into a centrifuge tube. After centrifuging the supernatant liquid is drained off and stored for analysis. If 2 ml. samples are taken for analysis the equivalent dry matter can be calculated from the equation:-

$$\text{Dry Matter Aliquot} = \frac{2 \times \text{Dry Matter (\%)}}{200 - \text{Dry Matter (\%)}}$$

Recoveries of volatile fatty acids from silage extracts

A mixture of known amounts of volatile fatty acids in sulphuric acid solution was added to an extract prepared from silage in which the amounts of volatile fatty acids had been determined. The recoveries of the added acids are shown in Table 21.

Determination of Lactic Acid using Ceric Sulphate

Apparatus

The specially designed steam distillation apparatus of Elsden and Gibson (1954), shown in Fig. 9 is used.

Reagents

(i) 0.05 N ceric sulphate in N sulphuric acid solution prepared from a stock solution of 0.5 N ceric sulphate in N sulphuric acid. The stock solution is standardised with ferrous ammonium sulphate.

(ii) 10 N sulphuric acid solution.

(iii) 0.5% ($\frac{W}{V}$) sodium bisulphite solution.

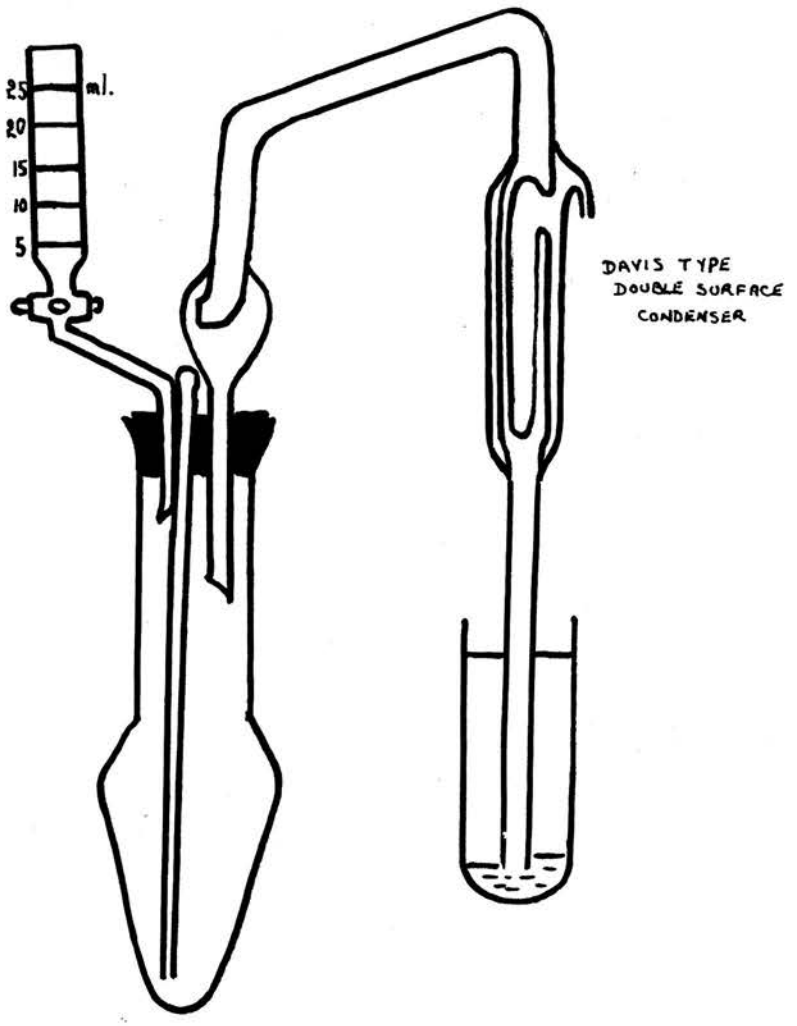

Fig. 9. Elsden and Gibson Apparatus for determination of lactic acid by oxidation with ceric sulphate.

(iv) $\frac{N}{10}$ and $\frac{N}{100}$ iodine solutions.

Procedure

The sample containing lactic acid is measured into the reaction vessel and sufficient 10 N sulphuric acid added to make the concentration of the solution normal. The reaction vessel is then attached the the apparatus. The receiving tube containing 2 ml. of 0.5% sodium bisulphite solution is attached in such a way that the tip of the condenser dips below the surface of the bisulphite solution. The reaction vessel is heated until the solution just boils. The steam is then turned on and the flow adjusted so that 15 - 20 ml./ minute of distillate are collected; the 0.05 N ceric sulphate is run into the reaction vessel at such a rate that each drop is decolorized before the next drop goes in. When a permanent yellow colour is obtained, further ceric sulphate is added to a total of 5 ml. When 15 ml. of distillate have been collected the receiver is lowered and the distillation continued until 20 ml. have been collected. The receiver is then placed in an ice water bath to cool.

The excess bisulphite in the receiver is removed by titration to the starch end-point with iodine solution and then the aldehyde bisulphite compound is destroyed by the addition of solid sodium bicarbonate solution. The sodium bisulphite released is titrated with $\frac{N}{100}$ iodine solution to the starch end point, the titration being carried out at 4 - 5°C.

Preparation of silage extract

A sample of 50g. of chopped silage is macerated with 250 ml. of

TABLE 22

Recoveries of known weights of lactic acid from a silage extract

Wt. of lactic acid added g	Wt. of lactic acid recovered g	Percentage recovery
0.156	0.150	96.42
0.632	0.619	97.98
1.031	1.012	98.15
1.538	1.512	98.30

CO_2 free water for 3 minutes. After filtering the extract through muslin an aliquot is taken for purification by treatment with copper sulphate and calcium hydroxide (1 ml. of 20% $CuSO_4$, $5H_2O$ and 1g. of $Ca(OH)_2$/5 ml. extract). Proteins are then removed by the addition of 1 ml. of 10% sodium tungstate solution and 1 ml. of 0.66 N H_2SO_4.

Recoveries of lactic acid from silage extracts

Lactic acid determinations were carried out on silage extracts prepared by the above method and determinations were then carried out on a mixture of 50 ml. of each extract with 50 ml. of solutions of lithium lactate in sulphuric acid solution. The recoveries of lactic acid obtained are shown in Table 22.

Determination of Alcohol

Apparatus

Conway standard microdiffusion units.

Reagents

(i) 0.4 N potassium dichromate in 10 N sulphuric acid solution.

(ii) $\frac{N}{10}$ sodium thiosulphate solution.

(iii) 3 M potassium iodide solution.

(iv) 1% starch solution.

(v) Fixative at 25°C:- vaseline.

Procedure

0.5 ml. of 0.4 N K_2Cr_2O7 is pipetted into the central chamber

TABLE 23

Recoveries of ethanol added to a silage extract

Ethanol added to extract g	Ethanol found in extract g	No. of determinations	Mean percentage recovery of ethanol
0.103	0.099	3	96.40
0.264	0.257	3	97.36
0.358	0.349	3	97.48
0.470	0.461	3	98.08

of a Conway microdiffusion unit. 1 ml. of sample is placed in the outer chamber and the lid is fixed on the unit with vaseline. After standing for at least 10 hours at 25°C the contents of the inner chamber are diluted with about 1 ml. of water, 0.5 ml. of 3 M KI solution are added with stirring and the liberated iodine titrated at once with $\frac{N}{10}$ sodium thiosulphate added from a microburette. The thiosulphate is added rapidly until nearly all the iodine has been reduced and then slowly until the solution is yellow-green in colour. A drop of 1% starch solution is then added and the titration completed to the point at which the deep blue starch iodine colour is replaced by the light blue-green colour of the chromic ion.

Preparation of silage extract

An aqueous extract of the silage is prepared by macerating 100g. of silage with 250 ml. of carbon dioxide free water.

Recoveries of ethanol from a silage extract

Known weights of ethanol were added to 300 ml. of silage extract in which had been shown to contain no alcohol and aliquots were taken for determination of alcohol. The percentage recoveries of ethanol thus obtained are shown in Table 23.

PART II

ENZYMOLYSIS OF GRASS HEMICELLULOSES

INTRODUCTION

The importance of the part played by carbohydrates in the formation of lactic acid in the ensilage process has long been recognized. Attention in the past was, however, necessarily restricted to the free sugars, mainly because of the limitations of existing analytical methods which made the determination of individual carbohydrates a matter of some difficulty. The introduction of paper chromatographic techniques to carbohydrate chemistry has resulted in a great increase in our knowledge of the carbohydrate constituents of grass. The grass carbohydrates may be divided conveniently into three groups:-

I. The free sugars and oligosaccharides.

II. The reserve polysaccharides.

III. The structural or cell-wall polysaccharides.

I. Laidlaw and Reid (1952) have found that glucose and fructose are the only monosaccharides present in grass while the most abundant free sugar is sucrose. In addition small quantities of the oligosaccharides raffinose and stachyose may occur in rye grass. The importance of the mono and disaccharides in ensilage is well known and Barnett (1954) has crudely represented the reactions by which they are converted to lactic acid by the following equations:-

$$C_6H_{12}O_6 \quad = \quad 2(C_3H_6O_3)$$

hexose lactic acid

$$C_{12}H_{22}O_{11} + H_2O = 4(C_3H_6O_3)$$

disaccharide — lactic acid

II. The main reserve carbohydrate occurring in grass is fructosan. Laidlaw and Reid (1951) have isolated a fructosan from perennial rye grass which is of the 2:6 linked levan type. Fructosan is extremely labile and Percival (1952) considers that it is probably quickly broken down to free sugar during ensilage. Several workers including Harwood (1954) have confirmed that this actually happens during the ensilage process. The supply of sugar released by fructosan decomposition may partially explain the observation made by Archibald (1953), McDonald and Purves (1956) and Langston et al (1958) that the sugar equivalent in the silage in the form of lactic, acetic and butyric acids is sometimes greater than can be accounted for by the free sugars in the original grass.

III. Cellulose is the chief structural carbohydrate in grass and there is at present little evidence to suggest that it undergoes breakdown to any great extent in the silage process. There occurs along with cellulose in the plant cell-wall a group of compounds known as hemicelluloses. These consist mainly of pentosans and hexosans and differ from cellulose in being soluble in dilute alkali solution and in being more easily hydrolysed by dilute acid. Wylam (1953) has found that after several months the pentose sugars arabinose and xylose appeared in laboratory silage. These were considered to be breakdown products of the hemicelluloses although, in the absence

of information on the quantity of hemicelluloses originally present, the possibility that they had been produced by bio-synthesis from carbon-containing compounds in the silage could not be overlooked. The development of a method for the determination of the cell-wall polysaccharides enabled Harwood (1954) to show that hemicellulose breakdown had occurred in laboratory silages after a period of two months. The breakdown was greater in silage inoculated with lactobacilli than in a control silage. As the pH of the silage was 6.6 and no free pentose sugars were found to be present there appeared to have been little or no acid hydrolysis of the hemicelluloses. Harwood concluded that the lactobacilli attack the pentosans after the depletion of free sugars and that the reaction takes place near the neutral point. The losses were 35% of the araban and 26 - 58% of the xylan. More recently McDonald et al (1960) have encountered considerable losses of hemicelluloses in low pH silages and small amounts of pentose sugars have been detected in the silages.

Wylam (unpublished data) has isolated from perennial rye grass an enzyme, capable of breaking down rye grass hemicellulose to pentose sugars, which had an optimum pH value between 5 and 6 but no quantitative studies were made. The same worker also found that B. pumilus and B. licheniformis produced traces of xylose from the hemicellulose preparation whereas B. polymyxa produced a little arabinose and glucose.

Macpherson, Wylam and Ramstad (1957) have studied the carbohydrate changes during ensilage using metabisulphite as

preservative and found that the silage contained more water soluble sugar than the original grass. This increase in sugar content was attributed to the hydrolysis of the cell-wall polysaccharides occurring at the low pH value.

Occurrence and molecular structure of the hemicelluloses

The nature of the association between the hemicelluloses and lignin in the plant cell-wall is not yet fully understood. The possibility that a chemical link exists between the hemicelluloses and lignin has been suggested by Harris, Sherard and Mitchell (1934) who were investigating the methylation of lignin in wood. These workers found that the hydroxyl groups of the lignin were not completely free for methylation until the wood had been submitted to a mild hydrolysis. Support for this view comes from the observation of Preece (1944) that pretreatments with hot alcoholic sodium hydroxide facilitate the extraction of hemicelluloses. Nelson and Scheurch, (1956) on the other hand have concluded that the relationship between hemicelluloses and lignin is a physical one.

Because of the association of hemicellulose with lignin it is customary to include a delignification procedure in the extraction of the hemicelluloses from grass. Early methods of delignification suggested by Cross and Bevan (1889) and Norris and Preece (1930) have been discarded because of the danger of hemicellulose degradation. Recent methods of delignification are based on the work of Jayme (1942) who introduced an acid-chlorite treatment which is claimed to effect complete removal of lignin with the minimum amount of degradation of

the hemicelluloses. After delignification the hemicellulose is usually removed from the grass by extraction with dilute alkali. The product thus obtained is not a single polysaccharide but a mixture of polysaccharides including xylans, arabans, araboxylans, galactans and glucans. A hemicellulose, isolated from delignified cocksfoot stems by Cairncross (1959) gave xylose, arabinose, glucose and galactose on acid hydrolysis and was shown to consist of a backbone of 1,4 linked xylopyranose residues with terminal arabinose groups linked to position 3 of the xylose residue. This hemicellulose may be taken as typical of many which have been isolated from various sources.

Present investigation

The question of what proportion of hemicellulose decomposition in silage is due to plant enzymes, bacterial action or chemical hydrolysis is not easily answered. Studies with silage give only the total decomposition and the individual effects are difficult to separate. For example any free sugar released by an enzyme or by chemical hydrolysis is likely to be immediately utilised by the bacteria present and transformed into an acid or acids. It cannot therefore be said that hemicellulose decomposition in which no pentoses are found in the free state is entirely due to bacterial action.

For this reason the present investigation was concerned with preparing a hemicellulose mixture from grass and using it for "in vitro" experiments to compare the effects of various enzymes extracted from grass. It was also intended to find out the effect of various pH

levels over a long period of time on the hemicellulose and to study the action of bacteria on samples of hemicellulose. Finally it was hoped that by studying microbe free grass, which undergoes little pH change during "ensilage" (Kemble 1956), the effect of any hemicellulose - splitting enzyme might be determined in the absence of interference from bacteria and chemical hydrolysis.

EXPERIMENTAL METHODS

Methods of Chemical Analysis

Determination of reducing sugars

Reducing sugars were determined by the titrimetic method of Somogyi (1945), the samples being heated for 20 minutes in a boiling water bath.

Determination of fructose

Fructose was determined by the Roe colorometric method as described by Wylam (1954).

Chromatographic separation of sugars

The separation of sugar mixtures was carried out by means of paper chromatography using Whatman No. 1 paper. The solvents used were:-

Solvent I. Ethyl acetate - acetic acid - water (3 - 1 - 3) (upper layer)

Solvent II. Benzene - butanol - pyridine - water (1 - 5 - 3 - 3) (upper layer)

Solvent I was used for the separation of fructose and arabinose and solvent II for the separation of glucose and galactose. After separation the papers were sprayed with aniline oxalate as described by Partridge (1949). When quantitative separations were carried out the positions of the sugars on the paper were shown by using side strips which were cut out and sprayed. The sugars on the main part of the paper were then located and cut out for elution.

Determination of the individual sugars after chromatographic separation

The chromatographic paper containing the sugar was cut into small pieces and was then shaken mechanically in a stoppered Pyrex test tube with 10 ml. of glacial acetic acid containing 0.2% benzidine and 2 ml. water. An aliquot sample of 6 ml. was then removed and heated in a boiling tube in a boiling water bath, the time of heating depending on the sugar under test. After cooling in running water for 5 minutes the intensity of the colour developed was measured on an EEL photoelectric colorimeter.

Methods of removing ions from solution

Negative and positive ions were removed from solution by electrodialysis between 'Permutit' ion-exchange membranes 'Permaplex C-20' and 'A-20'. Amberlite resins IR-120 (H) and IR-4B (OH) were also used for deionising solutions.

Preparation of Hemicellulose

Hemicellulose was prepared from a sample of perennial rye grass (Lolium perenne) which was representative of several cuts made in the Summer of 1958. The following method of extraction was used.

Preliminary extraction of the grass

Colouring matter and free sugars were removed by extraction with boiling 80% ethanol in a Soxhlet apparatus for 8 hours. The residue was then air dried and extracted with boiling water for 8 hours.

After filtering through muslin the residue was washed with acetone and air dried.

Delignification

Quantities of 25g. of the residue from the hot water extraction were mixed with about 800 ml. of water and the mixture was maintained at 70°C for 4 hours. During this treatment four additions of glacial acetic acid (2.5 ml.) and sodium chlorite (7.5g.) were made. The delignified grass was washed with water till free of chlorite, then with acetone and dried.

Extraction of hemicellulose

The delignified material (holocellulose) was shaken for 8 hours with N sodium hydroxide solution in a mechanical shaker and the resultant sludge was filtered through muslin. The residue was shaken for a further 8 hours with N sodium hydroxide solution and the filtrate added to that from the first extraction. After clarification by filtration through paper pulp, the combined extract was acidified to pH 5 with glacial acetic acid and the hemicellulose precipitated by the addition of two volumes of acetone. The hemicellulose was then washed by decantation with graded strengths of acetone and water until the strength of acetone was 95% ($\frac{v}{v}$). The hemicellulose was finally filtered, using a water pump, washed with acetone and ethanol and dried in a vacuum desiccator. The hemicellulose thus prepared had an ash content of 6.7%.

TABLE 24

Sugars produced by acid hydrolysis of purified hemicellulose

Sugar	Percentage of purified hemicellulose
Xylose	58.5
Arabinose	14.6
Glucose	6.1
Galactose	5.7

Purification of hemicellulose

The hemicellulose preparation was shaken with water at about 40°C and then cooled and centrifuged. The supernatant liquid was acidified to pH 5 with acetic acid and the hemicellulose reprecipitated with two volumes of acetone. The hemicellulose was washed with acetone as before, filtered and dried under vacuum. The ash content of the purified hemicellulose was 1.3%.

Quantitative analysis of hemicellulose

Hemicellulose (250 mg.) was hydrolysed by heating on a boiling water bath with N sulphuric acid solution for 4 hours under reflux. After cooling ribose (200 mg.) was added as reference sugar and the hydrolysate was neutralised with saturated barium hydroxide solution. After filtration, final traces of ions were removed in the electrodialysis apparatus and the solution was evaporated to a small volume and spotted onto a chromatogram paper. Sugars were eluted from chromatograms run in solvents I and II and their quantities determined by the benzidine method. The composition of the purified hemicellulose is shown in Table 24.

Preparation of Enzymes

Enzymes were prepared from freshly cut samples of perennial rye grass (Lolium perenne), Italian rye grass (Lolium italicum) and cocksfoot (Dactylis glomerata). All the samples were at the leafy stage of growth. The following method of preparation was used:

About 3½ lb. of the fresh grass were chopped into short lengths

with hand scissors, cooled to 0°C and mixed in the presence of ice and ice-cold water. About an eighth of the original (i.e. about 250g. fresh grass) was removed and extracted by stirring with about 2 litres of 0.25% sodium carbonate solution for 8 hours at 4°C. The mixture was filtered through muslin and centrifuged. The supernatant liquid was decanted and dialysed against running water for 4 days. Solid ammonium sulphate was then added with stirring to 75% saturation, (saturated = 700g/l) in order to precipitate the enzyme. The mixture was allowed to stand overnight at 4°C then the bulk of the supernatant liquid was decanted off and discarded. The remainder of the mixture was centrifuged and the precipitate dissolved in water and dialysed for 3 days against running water to remove final traces of ammonium sulphate. The dialysed solution was then centrifuged, to remove a small amount of precipitate which had settled out, and the enzyme obtained from the clear solution by freeze drying.

Enzymolysis of Hemicellulose Preparation

The activity of the enzymes extracted from grass was measured by the increase in reducing power produced by each enzyme in a hemicellulose solution. A range of times, temperatures and pH values similar to those encountered in the silage process was used. Experiments were planned according to a statistical design so that interactions, as well as the main effects, of time, temperature and pH might be studied. The design was a 3 x 3 x 3 factorial with four replications (carried out in two batches) and three levels of

temperature forming blocks. Tubes were made up containing:- 1 ml. of 1% purified hemicellulose solution, 1 ml. of enzyme solution containing 1 mg. of enzyme, 2 ml. of a phosphate-citric acid buffer solution, 0.25 ml. of chloroform and 0.25 ml. of toluene. Three levels of pH (4, 5 and 6) were used. The tubes were stoppered and randomized in wire baskets for incubation at 22°, 30° and 37°C. Blank determinations were carried out using 1 ml. of water instead of enzyme solution.

The reducing value of each solution was examined at intervals of 1, 2 and 3 days by centrifuging the contents of the tubes and withdrawing 1 ml. samples for determination of reducing sugar by the Somogyi method. By subtracting the reducing values of the blank determinations the production of reducing sugar due to the enzyme alone was measured. Samples of the solutions at each pH and temperature level were taken for bacteriological examination and were declared free from bacteria. (Kindly carried out by Mr R. Whittenbury of the Bacteriology Department, Edinburgh School of Agriculture.) The experiment was later repeated using temperatures of 43°, 50° and 57°C.

Long term effect of enzymes on hemicellulose

An experiment of a similar design to the first was performed using times of 1, 2 and 4 weeks. The quantities of reducing sugar in all the tubes were the same at the end of two and four weeks as at the end of 1 week showing that enzyme action had stopped after 1 week.

TABLE 25

Relative quantities of simple sugars, expressed as a percentage of total simple sugar, resulting from the enzymolysis of perennial rye grass hemicellulose for 1 week at 37°C

	Source of enzyme		
Sugar	Perennial rye grass	Italian rye grass	Cocksfoot
Xylose	42.6	47.3	60.1
Arabinose	44.0	40.1	26.9
Galactose	6.1	6.1	6.3
Glucose	7.2	6.5	6.7

TABLE 26

Reducing sugar (expressed as mg. xylose) resulting from the effect of pH on 10 mg. purified hemicellulose at three temperatures for a period of 3 months

pH	22°C	30°C	37°C
4	0.71	0.76	0.84
5	0.46	0.48	0.48
6	0.38	0.44	0.44

Chromatographic examination of products of enzymolysis

In order to determine the nature of the sugars present after enzymolysis 20 ml. quantities of 1% hemicellulose solution were incubated at 37°C for 1 week with 40 ml. of buffer solution at pH 6, 10 ml. of 0.1% enzyme solution and 2 ml. of chloroform and toluene. After centrifuging, each solution was deionized and evaporated to a small volume for spotting on to a paper chromatogram. Papers were run in solvents I and II and the quantities of reducing sugars on the papers were determined after elution by the benzidine method. The main sugars on the chromatogram were xylose, arabinose, glucose and galactose with traces of oligosaccharides. The results of the experiment are shown in Table 25.

Chemical Hydrolysis of Hemicellulose

Tubes containing 1 ml. of 1% hemicellulose solution were incubated at 37°C for a period of three months with buffer solutions having pH values of 4, 5 and 6, in order to find the amount of hemicellulose breakdown due to chemical hydrolysis. Tubes containing known amounts of xylose were also incubated to see if any sugar destruction took place.

Experiments with Micro-organism free grass

Two experiments were carried out using micro-organism free grass, supplied by Dr A. C. Stirling of the Bacteriology Department, Edinburgh School of Agriculture, in order to find out if any

hemicellulose breakdown occurred due to plant enzyme activity when the grass was "ensiled" under aseptic conditions. In the first experiment test tubes were packed with young timothy grass inside the sterile growth chamber, developed by Stirling (unpublished data), and stoppered with mercury seals before removal. The tubes were kept at 37° and the contents were examined after periods of 7 and 28 days for carbohydrate constituents. The contents of each tube were extracted immediately with boiling 80% ethanol and the free sugars in the extract were determined, after purification with cadmium hydroxide, by the Somogyi method. The total reducing value of the extract was also determined after hydrolysis of a sample with N sulphuric acid. The residue from the ethanol extraction was extracted with cold water and the fructose content of the extract was determined by Roe's method. The residue was hydrolysed with N sulphuric acid for 4 hours and the pentose sugars in the hydrolysate were determined by paper chromatography after neutralization and deionization. The weights of cellulose and lignin present were then determined in the residue.

The analysis of the carbohydrate constituents of the original grass was carried out by the same method.

In the second experiment only a limited amount of sterile grass was available and tubes were incubated at 37°C for one week only. In this case preweighed tubes were stoppered with cotton wool inside the growth chamber and weighed before replacing the cotton wool plugs with mercury seals. The freshly cut grass and grass after ensilage for 1 week were analysed for carbohydrate constituents as above.

In all the experiments with sterile grass samples of the grass were taken for bacteriological examination and were found to be free from micro-organisms.

RESULTS AND DISCUSSION

TABLE 27

Main effects of pH, time and temperature on the production of reducing sugar (mg. xylose) from 10 mg. of purified hemicellulose by enzyme from perennial rye grass

			L.S.D.				L.S.D.
P_1	P_2	P_3	(p = 0.01)	P_1	P_2	P_3	(p = 0.01)
0.381	0.827	0.964	0.039	0.326	0.853	0.887	0.019
t_1	t_2	t_3		t_1	t_2	t_3	
0.388	0.776	1.007	0.039	0.455	0.681	0.924	0.019
T_1	T_2	T_3		T_4	T_5	T_6	
0.636	0.750	0.788	0.028	0.681	0.698	0.682	0.016

Key

P_1 = pH 4
P_2 = pH 5
P_3 = pH 6

t_1 = 1 day
t_2 = 2 days
t_3 = 3 days

T_1 = 22°C
T_2 = 30°C
T_3 = 37°C
T_4 = 43°C
T_5 = 50°C
T_6 = 57°C

TABLE 28

Main effects of pH, time and temperature on the production of reducing sugar (as mg. xylose) from 10 mg. of purified hemicellulose by enzyme from Italian rye grass

			L.S.D.				L.S.D.
P_1	P_2	P_3	(p = 0.01)	P_1	P_2	P_3	(p = 0.01)
0.356	0.955	0.998	0.077	0.334	0.858	0.953	0.037
t_1	t_2	t_3		t_1	t_2	t_3	
0.478	0.819	1.033	0.077	0.525	0.725	0.814	0.037
T_1	T_2	T_3		T_4	T_5	T_6	
0.725	0.818	0.788	0.012	0.704	0.739	0.703	0.009

Key as in Table 27

TABLE 29

Main effects of pH, time and temperature on the production of reducing sugar (as mg. xylose) from 10 mg. of purified hemicellulose by enzyme from cocksfoot grass

			L.S.D.				L.S.D.
P_1	P_2	P_3	(p = 0.01)	P_1	P_2	P_3	(p = 0.01)
0.343	0.591	0.635	0.031	0.360	0.571	0.691	0.014
t_1	t_2	t_3		t_1	t_2	t_3	
0.320	0.510	0.719	0.031	0.321	0.561	0.739	0.014
T_1	T_2	T_3		T_4	T_5	T_6	
0.471	0.536	0.554	0.025	0.594	0.532	0.505	0.016

Key as in Table 27

RESULTS AND DISCUSSION

The effects of time, pH and temperature on the action of each of the grass enzymes on the hemicellulose preparations were studied statistically. An example of the type of calculation used is shown in Appendix III. The main effects of time, pH and temperature are shown in Tables 27, 28 and 29. L.S.D. (least significant differences) are given separately for the two ranges of temperatures studied for each enzyme. The amount of action was measured by determination of the amount of free sugars. Although this may not give a completely true picture of the breakdown that has taken place, it is justifiable in that production of free sugars is important in the ensilage process. A study of Tables 27, 28 and 29 shows that certain similarities in the characteristics of the three enzymes are obvious. Enzyme action increases with pH in every case with the optimum at pH6. In every case too the enzyme action increases significantly with time. In dealing with the main temperature effect it is necessary to treat each of the temperature ranges separately. In the range 22°- 37°C the enzyme from perennial rye grass showed greatest action at 37°C being significantly greater at the 1% probability level than the action at 30°C. The cocksfoot enzyme also showed greatest action at 37° but was significant at the 5% level of probability. The enzyme isolated from Italian rye grass on the other hand had an optimum temperature of 30°C.

In the higher temperature range (43 - 57°C) the enzymes from both perennial and Italian rye grasses had an optimum temperature of 50°C

TABLE 30

Interactions of pH, time and temperature on the production of reducing sugar (as mg. xylose) from 10 mg. of purified hemicellulose by enzyme from perennial rye grass

	P_1	P_2	P_3
t_1	0.151	0.487	0.526
t_2	0.398	0.895	1.040
t_3	0.592	1.102	1.331

	P_1	P_2	P_3
t_1	0.316	0.560	0.589
t_2	0.330	0.852	0.861
t_3	0.433	1.130	1.211

	P_1	P_2	P_3
T_1	0.352	0.717	0.838
T_2	0.413	0.860	0.977
T_3	0.376	0.904	1.077

	P_1	P_2	P_3
T_4	0.336	0.816	0.892
T_5	0.344	0.864	0.886
T_6	0.300	0.863	0.883

	t_1	t_2	t_3
T_1	0.348	0.684	0.876
T_2	0.395	0.794	1.061
T_3	0.421	0.851	1.085

	t_1	t_2	t_3
T_4	0.402	0.679	0.963
T_5	0.465	0.685	0.943
T_6	0.399	0.679	0.868

L.S.D. ($P = 0.01$) = 0.067

L.S.D. ($P = 0.05$) = 0.051

L.S.D. ($P = 0.01$) = 0.033

L.S.D. ($P = 0.05$) = 0.025

Key as in Table 27

TABLE 31

Interactions of pH, time and temperature on the production of reducing sugar (as mg. xylose) from 10 mg. of purified hemicellulose by enzyme from Italian rye grass

	P_1	P_2	P_3
t_1	0.181	0.605	0.655
t_2	0.365	1.052	1.042
t_3	0.522	1.241	1.337

	P_1	P_2	P_3
t_1	0.194	0.650	0.734
t_2	0.372	0.820	0.985
t_3	0.437	1.106	1.141

	P_1	P_2	P_3
T_1	0.307	0.917	0.951
T_2	0.400	0.997	1.057
T_3	0.362	0.985	1.019

	P_1	P_2	P_3
T_4	0.345	0.821	0.943
T_5	0.350	0.876	0.993
T_6	0.306	0.879	0.922

	t_1	t_2	t_3
T_1	0.467	0.725	0.983
T_2	0.517	0.864	1.080
T_3	0.451	0.877	1.037

	t_1	t_2	t_3
T_4	0.470	0.731	0.911
T_5	0.552	0.751	0.915
T_6	0.555	0.695	0.852

L.S.D. ($P = 0.01$) = 0.131

L.S.D. ($P = 0.05$) = 0.097

L.S.D. ($P = 0.01$) = 0.082

L.S.D. ($P = 0.05$) = 0.065

Key as in Table 27

TABLE 32

Interactions of pH, time and temperature on the production of reducing sugar (as mg. xylose) from 10 mg. of purified hemicellulose by enzyme from cocksfoot grass

	P_1	P_2	P_3
t_1	0.185	0.352	0.423
t_2	0.341	0.564	0.660
t_3	0.504	0.766	0.889

	P_1	P_2	P_3
t_1	0.196	0.359	0.408
t_2	0.359	0.608	0.717
t_3	0.327	0.744	0.947

	t_1	t_2	t_3
T_1	0.269	0.519	0.595
T_2	0.390	0.564	0.653
T_3	0.370	0.569	0.725

	P_1	P_2	P_3
T_4	0.376	0.610	0.766
T_5	0.359	0.573	0.664
T_6	0.347	0.528	0.642

	t_1	t_2	t_3
T_1	0.293	0.471	0.649
T_2	0.345	0.532	0.731
T_3	0.322	0.559	0.779

	t_1	t_2	t_3
T_4	0.339	0.581	0.833
T_5	0.328	0.558	0.710
T_6	0.296	0.545	0.676

L.S.D. ($P = 0.01$) = 0.053

L.S.D. ($P = 0.05$) = 0.041

L.S.D. ($P = 0.01$) = 0.024

L.S.D. ($P = 0.05$) = 0.018

Key as in Table 27

TABLE 33

Weights (in mg.) of reducing sugars (as xylose) produced after incubation of hemicellulose for 7 days with grass enzymes

pH	Source of enzyme								
	Perennial rye grass			Italian rye grass			Cocksfoot		
	22°C	30°C	37°C	22°C	30°C	37°C	22°C	30°C	37°C
4	1.20	1.20	0.90	0.95	1.15	1.18	1.11	1.16	0.98
5	1.70	1.79	2.16	1.73	1.85	2.03	1.60	1.60	1.74
6	2.08	2.18	2.28	1.85	2.05	2.08	1.58	1.65	1.72

although their activity at this temperature range was less than that at their temperature optimum in the lower temperature range. The cocksfoot enzyme had an optimum temperature of 43°C and its action at this temperature was greater than at its optimum in the lower range.

The interactions between the three main effects are given separately for each enzyme in Tables 30, 31 and 32. The most interesting interaction was that between temperature and pH. In the case of the enzymes from the Italian and perennial rye grasses the activity of the enzyme was significantly lower at 37°C and pH 4 than at 30°C at the same pH. This interaction of high temperature and low pH value is also observable to a significant extent in the temperature range 43° - 57°C.

The results in Table 33 show that enzyme activity has continued for a period longer than the three days and the failure to observe any further increase in reducing value after 7 days showed that enzyme action was complete at the end of this time. The greatest amount of reducing sugar has been produced by the enzyme from perennial rye grass at a pH value of 6 and a temperature of 37°C.

The results of the quantitative chromatographic analysis of the products of enzymolysis of the hemicellulose incubated at pH 6 and 37°C are of interest when compared with the results of the complete hydrolysis effected by mineral acid (Table 24). The latter results show that the arabinose-xylose ratio in the original hemicellulose is approximately one to four. In the enzyme digests, however, the ratio

TABLE 34

Percentage Composition of Fresh Sterile Grass

	Experiment 1			Experiment 2	
	As ensiled	After 7 days	After 28 days	As ensiled	After 7 days
Dry Matter	12.18	11.76	11.66	11.85	11.65
Nitrogen	0.76	-	-	0.77	-
Reducing Sugar in 80% ethanol extract (as glucose)	0.083	0.486	0.489	0.081	0.501
Total sugars in 80% ethanol extract (as glucose)	0.321	0.489	0.511	0.311	0.521
Fructosan	Nil	Nil	Nil	Nil	Nil
Sugars in N H_2SO_4 extract as (xylose)	1.04	0.87	0.85	1.01	0.85
Ratio Xylose: Arabinose	2.8:1	2.1:1	2.1:1	2.85:1	2.65:1

is approximately one to one in the case of perennial and Italian rye grass enzymes and about one to two in the case of cocksfoot. The increase in the arabinose to xylose ratio seems to indicate that arabinose has been split off preferentially from the xylan chain by the enzyme. As no attempt to purify or fractionate the enzyme was made, it cannot be said whether this effect was due to a specifically arabinose-splitting enzyme or merely to the greater accessibility of the arabinose residues because of their position as side chains to the main xylan chain.

The results in Table 26 show the effect of pH on the hemicellulose over a period of three months. The fairly high quantities of sugars produced at pH 4 suggest that considerable breakdown due to chemical hydrolysis may occur in low pH silages stored for a long period of time. Unfortunately, no chromatographic separations were carried out on the products of hydrolysis so that there is no information available on the proportions of arabinose and xylose liberated.

The results of the investigation with sterile grass show that a small amount of hemicellulose breakdown has occurred (Table 34). As the ensiled material was found to be free from micro-organisms and the pH of the sample did not fall below 6.8 the breakdown must have been due to plant enzyme activity. Chromatographic separation of the sugars present in the alcohol extract of the ensiled material showed that the main sugars present were glucose, fructose, xylose and a little arabinose. It is clear from a study of Table 34 that nearly all the non-reducing sugars in the ethanol extract of the "ensiled"

material have been broken down to reducing sugars. The xylose and arabinose, which are not usually present to any great extent in the ethanol extract, must have come from the hemicellulose fraction. The small amount of starting material used made an accurate determination of the xylose to arabinose ratio in the ethanol extract difficult but it was calculated for the sugars separated in the N sulphuric acid extract. From the results obtained there is some evidence that the loss of xylose was greater than that of arabinose, a result which is contrary to the findings in the previous experiments. The extent of the hemicellulose breakdown is also slightly less than was encountered over a similar period in the experiments with enzymes and the hemicellulose preparation.

One of the objects of the present investigation was to attempt to grow lactic acid bacteria on hemicellulose with a view to isolating any bacterial enzyme which could bring about hemicellulose breakdown. Work was carried out in co-operation with the Bacteriology Department and strains of several lactic acid bacteria, isolated from grass and silage, were investigated. All the bacteria could grow on a basal medium containing sugar but failed to grow on the same medium when the sugar was replaced by hemicellulose. A list of the bacteria examined is given in Appendix IV. These results do not support the theory of Harwood (1954) that, in the absence of sugar, lactobacilli attack hemicelluloses.

In view of the observation of Wylam (unpublished data) that B. pumilus, B. licheniformis and B. polymyxa produced traces of reducing

sugars from hemicelluloses, mixtures of cell suspensions of these aerobic bacteria with hemicellulose solution were examined manometrically for oxygen uptake. In no case was there an increase in oxygen uptake over that of control samples which contained no hemicellulose, showing that no growth had taken place.

The presence of a plant enzyme which can degrade insoluble cell-wall polysaccharides is of obvious importance in the ensilage process. The optimum conditions of pH and temperature of the three enzymes studied in this investigation are likely to be easily fulfilled during the first few days of ensilage when the pH of the mass is in the region of 6. It is interesting to note that in the "in vitro" experiments performed in this investigation the enzyme continued to function at temperatures up to 57°C so that the enzyme should stand up well to the changes of temperature encountered in the ensilage process. The activity of the enzyme, however, is likely to diminish when the pH drops to about 4 and the loss of activity will be greater at higher temperatures.

The presence of an enzyme which can attack hemicelluloses is likely to be of considerable importance in grass which is wilted before ensilage and will be specially important if the soluble carbohydrate content of the grass is low. McDonald (1956) found that silage prepared from wilted grass had a slightly higher soluble sugar content than silage prepared from unwilted material. The nature of the soluble sugars was not determined so that it is not possible to say whether this was due to the presence of pentose sugars released

during the wilting process.

Although no bacteria were found which could utilise hemicellulose as a source of energy the possibility exists that bacteria may be able to attack hemicellulose which has been degraded to shorter chain lengths by the action of plant enzyme or by chemical hydrolysis or by a combination of the two processes. The presence of pentose containing oligosaccharides was noticed on the paper chromatograms run on the digests from the enzyme experiments, but these were not determined quantitatively. The investigation of this aspect of the problem seems to be a possible subject for further study.

SUMMARY

1. The production of reducing sugars, resulting from the incubation of a rye grass hemicellulose preparation with enzymes extracted from perennial and Italian rye grasses and cocksfoot, was measured over a range of temperatures, pH values and times.

2. A statistical analysis of the results showed that each enzyme had an optimum pH of 6 while the optimum temperatures varied from 30°C for the enzyme from Italian rye grass to 43° for the cocksfoot enzyme. In every case the enzyme activity continued to act over a period of three days. There was a significant interaction between high temperature and low pH which tended to suppress the enzyme activity.

3. The effect of three pH levels on the hemicellulose was measured after an incubation period of three months at three temperatures. The greatest production of reducing sugar was found at the lowest pH (4) and at the highest temperature (37°C).

4. Samples of sterile grass were ensiled aseptically in test tubes at 37°C and the carbohydrate constituents determined after periods of 7 and 28 days. In every case small quantities of pentose sugars were found in the "silage" and losses of the hemicellulose fraction had also taken place.

5. Attempts to grow certain strains of lactic acid bacteria using hemicellulose as an energy source were unsuccessful. All suspensions of aerobic bacteria also failed to have any effect on the hemicellulose.

APPENDIX III

Example of Method of Statistical Calculation

The calculation was made using the figures obtained for the production of reducing sugar per 1 ml. of solution in the experiment ~~with the experiment~~ with the enzyme from perennial rye grass in the range 22.37°C. All figures have been multiplied by 1000 to eliminate decimals.

Factor	Sum of Squares	Degrees of Freedom	Variance	F	Significance
Blocks (T)	27704	2	13852	164.5	1%
Error (T)	758	9	84.2		
Sub-blocks	28462	11			
Main P	419039	2	209520	815	1%
Main t	440520	2	220260	857	1%
Inter Pt	26774	4	6694	26.0	1%
Inter PT	9780	4	2445	9.5	1%
Inter tT	4966	4	1242	4.83	1%
Error	20569	80	257.1		
Total	950110	107			

Main T L.S.D. (P = 0.01) = $\sqrt{84.2 \times 2 \times 36} \times 3.25 = 253$

Main P and t L.S.D. (P = 0.01) = $\sqrt{257.1 \times 2 \times 36} \times 2.58 = 351$

Inter Pt
Inter PT
Inter tT

L.S.D. (P = 0.01) = $\sqrt{257.1 \times 2 \times 12} \times 2.58 = 203$

L.S.D. (P = 0.05) = $\sqrt{257.1 \times 2 \times 12} \times 1.96 = 154$

APPENDIX IV

Lactic acid bacteria examined (sec.p.75):-

Lactobacillus *plantarum*	-	20 strains
Lactobacillus *casei*	-	15 strains
Lactobacillus *brevis*	-	30 strains
Lactobacillus *buchneri*	-	20 strains
Lactobacillus *fermenti*	-	5 strains
Leuconostoc *mesenteroides*	-	100 strains
Leuconostoc *dextranicum*	-	4 strains

APPENDIX V

Analytical Methods

1. Determination of reducing sugars - Somogyi method

Reagents:- N potassium iodate

2.5% potassium iodide

2 N sulphuric acid

$\frac{N}{10}$ sodium thiosulphate

Standard copper reagent containing:- 28g. anhydrous disodium hydrogen phospate, 100 ml. N sodium hydroxide solution, 40g. Rochelle salt, 8g. copper sulphate (pentahydrate) and 180g. anhydrous sodium sulphate in 1 litre of solution. A known volume of N potassium iodate is then diluted with the copper reagent in a graduated flask, the amount of iodate used being dependent on the concentration of sugar to be determined.

Method:-

5 ml. of the sugar solution under test are pipetted into a large boiling tube and 5 ml. of Somogyi reagent containing potassium iodate are added. The tube is lightly stoppered and heated in a boiling water bath for 20 minutes. After cooling in running water for 5 minutes, 2 ml. of 2.5% potassium iodide solution are added down the side of the tube and then 2.5 ml. of 2 N sulphuric acid are added quickly with agitation to effect immediate mixing. The liberated iodine is then titrated with $\frac{N}{200}$ sodium thiosulphate, prepared from

the stock $\frac{N}{10}$ solution, using 1% starch solution as indicator. Blank determinations, using 5 ml. of water instead of sugar solution, are carried out each time.

ml. sodium thiosulphate equivalent to sugar = ml. for blank - ml. for back titration

mg. sugar in 5 ml. solution = ml. thiosulphate x factor

The following factors were determined with pure sugar solutions:-

Glucose	0.134	Fructose	0.139
Xylose	0.134	Arabinose	0.145

Determination of fructose - Roe's method

Reagents:- "Acid reagent" containing 130g. glycerol, 100 ml. conc. hydrochloric acid, 4.5 mg. of copper sulphate (pentahydrate) and 50 ml. water.

0.45% solution of resorcinol in water.

Method

To 5 ml. of acid reagent are added 1 ml. of 0.45% resorcinol solution and 2 ml. of test solution. After mixing, the tube is lightly stoppered and heated in a boiling water bath for exactly 12 minutes. The tube is then cooled and the contents transferred to the cell of a photoelectric absorbtiometer for measurement of the colour produced, a violet filter being used. A blank determination using 2 ml. of water is read simultaneously the difference in colour intensity being due to fructose.

A series of known concentrations of fructose are used to construct a standard graph from which unkown quantities of fructose may be read after development of colour.

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